ESTROGENIC PROPERTIES OF SORGHUM PHENOLICS:

POSSIBLE ROLE IN COLON CANCER PREVENTION

A Dissertation

by

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ABSTRACT

Consumption of whole grains has been linked to reduced risk of colon cancer. This study determined estrogenic activity of sorghum phenolic extracts of different phenolic profiles and identified possible estrogenic compounds in sorghum *in vitro*, as well as evaluated the potential of estrogenic sorghum phenolic extracts to prevent colon carcinogenesis *in vivo*. The thermal stability of sorghum 3-deoxyanthocyanins was also studied, to determine their suitability as functional food colorants.

White and TX430 (black) sorghum extracts showed estrogenic activity in cell models predominantly expressing estrogen receptor-α (ERα) or ERβ at 5 and 10 μg/mL, respectively. The same treatments led to induction of apoptosis in cells expressing ERβ. The red TX2911 sorghum did not possess these activities. Compositional analysis revealed differences in flavones and flavanones. Flavones with estrogen-like properties, i.e. luteolin and apigenin, were detected in White and TX430 (black) sorghum extracts, but not in red TX2911 extract. Naringenin, a flavanone known to antagonize ERα signalling, was only detected in the red TX2911 extract. Additional experiments with sorghum extracts of distinct flavones/flavanone ratio, as well as with pure apigenin and naringenin, suggested that flavones are the more potent ERβ agonists in sorghum. On the other hand, 3-deoxyanthocyanins were probably not estrogenic.

Estrogenic white and black sorghum phenolic extracts (fed at 1% level in the diet) reduced the number of azoxymethane induced colon premalignant lesion (aberrant

crypt foci) by 39.3% and 14.7%, respectively, in ovariectomized mice. Further studies are needed to elucidate the protective mechanisms induced by these sorghum extracts.

Sorghum 3-deoxyanthocyanins retained good color stability after 30 minutes of heat treatment at 121 °C under pressure: More than 80% of color retained in pH 1 and 2 HCl and citric acid solutions, and 39-84% retained from pHs 3-7. Formic acid negatively affected the color stability at pH 1 and pH 2 due to its reducing capacity. Methoxylation decreased the thermal stability of 3-deoxyanthocyanins. The heat stability of 3-deoxyanthocyanins indicates good potential for food use.

Overall, the inherent estrogenic activity of specific sorghum phenolic extracts is a likely mechanism for colon cancer prevention. Further studies are needed to assess physiologically relevant dietary level of sorghum phenolics for prevention of colon cancer, and effect of food processing on the activity and bioavailability of the chemopreventive components.

DEDICATION

献给

我挚爱的家人

我的父亲 杨显宇先生 母亲 温磊女士

以及

我的丈夫 陈明先生

TO MY BELOVED FAMILY

My father Mr. Xianyu Yang and mother Ms. Lei Wen

as well as

My husband Mr. Ming Chen

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CHAPTER I

INTRODUCTION

Colon cancer is one of the most common types of cancers in the United States and around the world. It accounts for around 9% of all cancer incidences and is the most prevalent diagnosed malignancy in the digestive tract (*1, 2*). Many factors have been associated with lower colon cancer risk, for example, consumption of whole grains (*3*), fruits and vegetables (*4, 5*), consumption of soybeans and related products (*6*), use of hormone replacement therapy (*7*), etc. The female hormone estrogen is considered protective against colon cancer because observational studies find lower incidence of colon cancer in females compared with males (*8, 9*), in premenopausal women compared with post-menopausal women (*10*), and in post-menopausal women who used hormone or estrogen replacement therapies (*7*). A plausible mechanism is estrogen-induced protective activities mediated through estrogen receptor-β (ERβ), the predominant estrogen receptor in the colon; e.g., via apoptosis in damaged colonocytes (*11*) and activation of tumor suppressor gene *p53 (12)*. However, the utilization of estrogen replacement therapy as a chemopreventive strategy remains controversial because elevated levels of estrogen is associated with higher risk of female reproductive system cancers, especially breast cancer (*7, 13*).

Phytoestrogens are a group of plant derived polyphenolic compounds which have similar physiological function as estrogen (17β-estradiol). They have shown benefits in colon cancer prevention but were not associated with the risk of traditional hormone

replacement therapy (*14*). Phytoestrogens are widely distributed in the plant kingdom but only a few major ones are present in human diet, which include isoflavones from legumes and lignans from whole grains (*15*).

Sorghum is an important cereal grain in many arid and semi-arid areas around the world (*16*). Consumption of sorghum was related to reduced incidence of esophageal cancer (*17*). Bran of black and tannin sorghum in the diet reduced the formation of premalignant lesions in the colon of rats treated with carcinogen (*18*). Our previous studies showed that phenolic extracts of sorghum inhibited the growth of human esophageal (OE33) and colon (HT-29) adenocarcinoma cell growth *in vitro*, with IC_{50}s (concentrations to inhibit 50% cell growth) ranging from 49.7-883 μg/mL (*19*). Compared with phenolic extracts from nuts (*20*) and grapes (*21*), sorghum extracts required lower concentrations to inhibit HT-29 cell growth. The mechanisms of chemoprevention and the active components are still unknown.

Sorghum grains of different genotypes are good sources of different groups of phenolic compounds (*22*). Sorghum grains with a pigmented testa under the pericarp accumulate high amounts of condensed tannins (proanthocyanidins) (*23*). Based on pericarp color, non-tannin containing sorghum grains can be divided into 3 main categories: White grains with the least amount of phenolics, red (including the black) grains with the highest amount of 3-deoxyanthocyanins, and lemon-yellow grains with high amounts of flavanones (e.g. naringenin) (*24, 25*). Sorghum secondary plant color (i.e. tan, red or purple) also influences the phenolic compositions of sorghum grains: Tan plants accumulate higher amounts of flavones (e.g. apigenin) than red/purple plants;

while red/purple plants accumulate higher amounts of 3-deoxyanthocyanins (*24, 25*). All sorghum grains contain phenolic acids (*26*).

Among the phenolic compounds present in sorghum, 3-deoxyanthocyanins are a unique group, as sorghum is the only edible source of these pigments. Compared to anthocyanins, 3-deoxyanthocyanins do not possess the C-3 substitution in the C-ring, and are more stable to pH changes (*27, 28*) and the bleaching effects of common food additives (*29*). These properties suggest 3-deoxyanthocyanins are suitable for food colorants. However, the effect of thermal processing on stability of 3-deoxyanthocyanins are still unknown. In addition to their color stability advantages, the 3-deoxyanthocyanins are more potent against human colon cancer cell growth *in vitro* than anthocyanins (*30*). In our previous *in vitro* study, black sorghum grain extract (highest in 3-deoxyanthocyanins) was the strongest cancer cell growth inhibitor among all non-tannin sorghums. The 3-deoxyanthocyanins are also capable of inducing quinone oxidoreductase (a phase II) activity in murine hepatoma cells. This indicates 3-deoxyanthocyanins possess chemopreventive potential that anthocyanins may not have. However, the chemopreventive mechanisms of 3-deoxyanthocyanins are unknown.

Many cellular signaling pathways have been suggested as mechanisms for protection against colon cancer development. Besides antioxidant and anti-inflammatory capacity, estrogenic activity and associated activation of ERβ is recognized as a chemopreventive pathway that is important in the early stages of colon carcinogenesis. Among sorghum polyphenols, flavones and flavanones are recognized as estrogenic (*31*). The 3-deoxyanthocyanins may also possess estrogenic activity based on their

structural similarity to estrogenic flavonoids (lack of C-ring substitution). This hypothesis needs to be investigated to determine the potential of 3-deoxyanthocyanins to activate ERβ, which is relevant to gastrointestinal tract cancer prevention.

The overall goal of this study is to evaluate estrogenicity as a potential chemopreventive mechanism of sorghum phenolics. This would lead to targeted utilization of specific sorghum varieties or compounds for cancer prevention, especially cancers of the gastrointestinal tract. The hypothesis is that sorghum phenolics possess estrogenic activity hence are capable of inducing apoptosis and other protective activities in the colon. Condensed tannins were not included in this study because their large molecular weight and spatial configuration likely excludes them from binding to estrogen receptors. The thermal stability of 3-deoxyanthocyanins, the most unique flavonoids in sorghum, was also studied since this property reflects the potential to use them as functional/chemopreventive food colorants.

The objectives of this study were:

1. To determine the estrogenic activity of sorghum grain phenolic extracts by *in vitro* methods;
2. To identify possible estrogenic compounds in sorghum grain phenolic extracts;
3. To determine the ability of estrogenic sorghum grain phenolic extracts to prevent azoxymethane induced colon carcinogenesis in ovariectomized mice;
4. To determine thermal stability of sorghum 3-deoxyanthocyanins.

CHAPTER II

LITERATURE REVIEW

Prevalence of Colon Cancer

Colon cancer is the third most prevalent type of cancer in the US, with 102,480 new cases and 50,830 deaths expected in 2013 (*1*). Colon cancer is also a common type of cancer worldwide, ranked as third most diagnosed cancer in males (663,600 new cases) and second in females (570,100 new cases), as well as the most commonly diagnosed malignancy in the digestive tract (*2*). In 2008, over 1.2 million new cases and 608,700 deaths were estimated to have occurred around the world. The incidence of colon cancer in developed countries, i.e. Australia and New Zealand, Europe, and North America, is among the highest in the world; the incidence in Africa and Southeastern Asia is the lowest. In the past decade, the incidence of colon cancer in developed countries has been stabilizing or decreasing, mostly due to early detection and removal of precancerous lesions through annual colon cancer screening (*32, 33*). On the other hand, the incidence in some areas where colon cancer used to be low, such as several countries in Eastern Asia and Eastern Europe, have increased (*33, 34*). Adopting to Western diet is believed to be a major contributor to such trend (*33, 34*).

Colon Carcinogenesis

Colonic carcinogenesis involves multi-step pathologic changes which turn normal colonic epithelial cells into invasive carcinoma (Figure 1) (*35, 36*). The process

can be roughly divided into three main stages, initiation, promotion and progression. The complete process can take decades and is related to mutations of multiple genes regulating cellular functions. Colon cancer may be sporadic, hereditary or as a result of inflammatory bowel disease (IBD) (*37*).

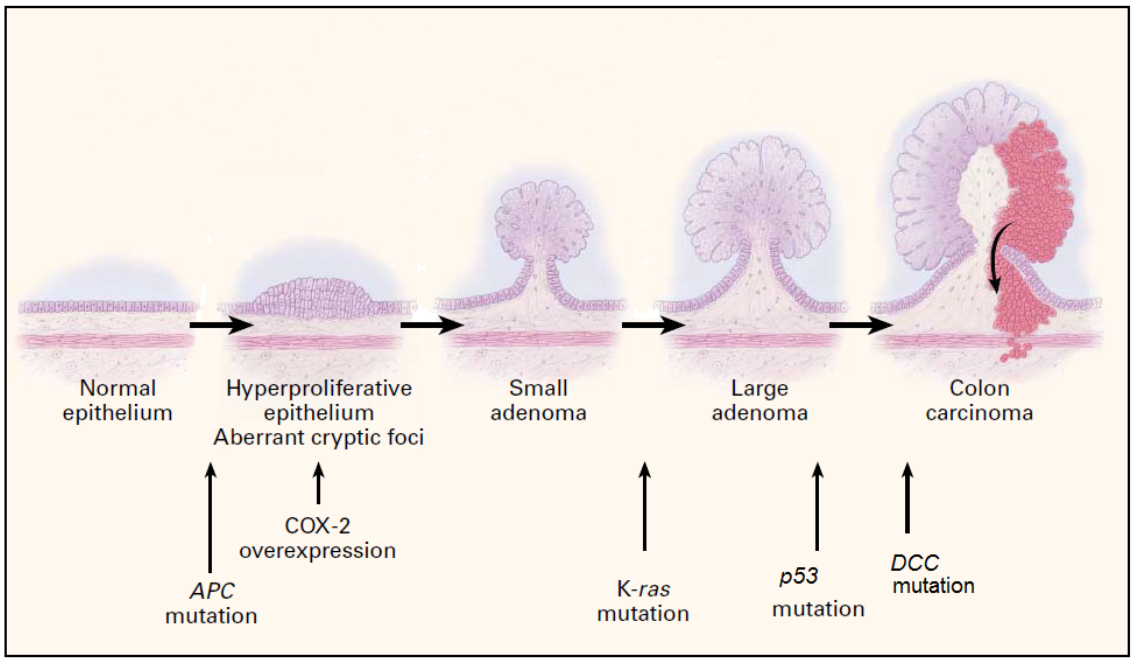


Figure 1. A simplified illustration of colon carcinogenesis with major pathological events and gene mutations. Figure was adapted with changes from Jänne, P.A. & Mayer, R.J. (*35*) with information from Kinzler, K.W. & Vogelstein, B. (*36*).

The initiation step starts with damage to DNA and results in loss of tightly maintained balance between apoptosis and proliferation/differentiation of normal colonic epithelium (colonocytes) (*37*). Colonic crypts are U-shape units of colonic mucosa. Stem

cells are located at the bottom of the crypts and divide slowly. Daughter cells are gradually pushed up along the sides of the crypts and differentiated into cells with various morphologies and functions. Cells moved to luminal surface of crypts become epithelial cells. The ones reaching the uppermost part undergo apoptosis and are slough off without further differentiation or replication (*38, 39*). During normal crypt development, one cryptic cell with genetic or epigenetic changes (mutated/damaged DNA) can develop resistance to apoptosis signal upon arrival at the uppermost part of the crypt and keep proliferating (Figure 1). The *APC* (adenomatous polyposis coli) gene mutation and associated overexpression of mutant β-catenin are commonly found in this initiation stage, causing increased proliferation and decreased apoptosis (*40*). Mutated *APC* gene could lead to mutation of k-*ras*, one of the oncogenes (*41*). The mutated forms of k-*ras* stimulate cell proliferation, transformation and differentiation (*42*), then as a result build up more resistance to apoptosis (*38, 40*).

The crypt with pre-cancerous properties is called aberrant crypt foci (ACF) and has been widely used as a biomarker of colon carcinogenesis in chemoprevention studies (*38, 43-45*). These ACFs are characterized by crypts with altered luminal openings, thickened epithelia and being larger than the adjacent normal crypts (*46*). Figure 2 shows examples of ACFs in animals and humans (*38*).

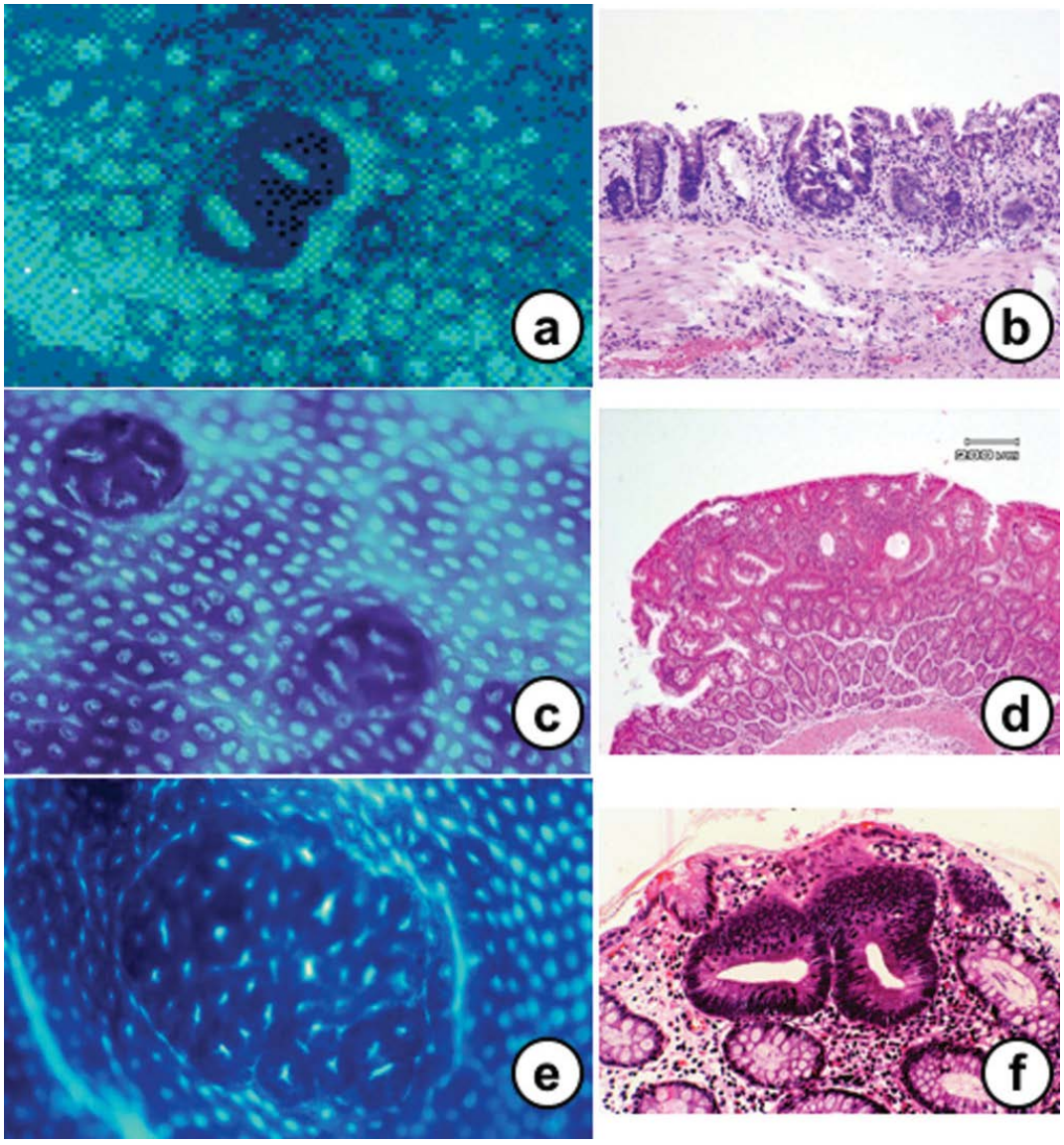


Figure 2. Images of colonic aberrant crypt foci (ACF) found in mouse (a and b), rat (c and d) and human (e and f). Methylene blue staining is capable of identifying ACF (a, c and e). Adapted from Tanaka et al. (*38*).

The promotion stage of colonic carcinogenesis mostly results in development of adenoma (well-demarcated lumps of epithelial tumor cells) from the hyperproliferative colon epithelium (Figure 1). Though most of them remain benign, a small fraction of

these lesions may evolve into malignancy (*38, 47*). The promotion stage transforms small adenomas to large adenomas and finally infiltrating carcinomas (Figure 1). The genetic events in these two stages include mutations of oncogenes (such as k-*ras*) and mutator genes (DNA mismatch repair genes, such as *MLH1* and *MSH2*), inactivation of tumor suppressor genes (such as *APC*, *p53* and *DCC* (deleted in colorectal carcinoma)) and disturbances of DNA methylation and microsatellite stability (*38, 48*).

Discovery of ACFs, removal of small benign adenomas and other pre-malignant lesions are effective in early detection of colon cancer reducing the probability of onset of the diseases. However, the required annual endoscopic screening and associated medical technique are not ubiquitously accessible and/or affordable. The estimated mean charge per discharge inpatient of colon cancer increased from $25,289 in 1998 to $51,569 in 2007; while the mean cost for each inpatient rose from $12,124 to $16,688 (a 37.6% increase) in that decade (*49*). The cost of cancer is also increasing on public health expenditures side. It is predicted that by 2020, the average colorectal cancer-related medical care cost will increase by 42.3%, to approximately $14.02 billion, in the US (*50*). On the other hand, prospective studies suggest as much as 70% of current colon cancer burden could be prevented by moderate modifications in dietary pattern (reduce red meat and increase poultry/fish consumption; adequate consumption of fruits, vegetables, whole grains and fiber) and lifestyle (avoid smoking and heavy alcohol use, maintain a healthy weight, and moderate level of physical activity) (*51, 52*). Hence the ultimate goal of "fighting" colon cancer should be primary prevention, which is of importance in improving the quality of lives and reducing health care expenditure.

Chemoprevention entails protective actions, including retarding, blocking or even reversing carcinogenesis (*53*). Practically, this strategy uses natural or synthetic chemical substances to reduce the risk of developing cancer, or to reduce the chance that cancer will recur. In colon cancer prevention, restoring the balance between apoptosis and proliferation, as well as reducing the formation of ACFs are used as major strategies (*38, 54*).

Diet and Risk of Colon Cancer

Dietary Factors Related to Increased Colon Cancer Risk

The majority of, though not all, evidence from epidemiological and observational studies suggest that diets high in saturated fat (*55*), red (*56*) and processed meats, highly refined grains/starches and added sugar (*57*) are related to higher risk of colorectal cancer.

Dietary saturated fat, red and processed meats cause increased concentration of bile acids in the bowel content, which can interfere with the balance between proliferative and apoptotic cells (*56*). In addition, the metabolites of bile acids, e.g. secondary bile acid deoxycholic acid, are capable of destroying cytoplasmic membrane in epithelial cells of the colon, which indirectly results in increased cell proliferation and higher sensitivity to mutagenic factors (*58*).

Consuming diets high in saturated fat, red and processed meats, refined grains and added sugar stimulate the secretion of insulin thus result in increased level of free

IGF-1 (insulin-like growth factor-1), which may lead to increased cell proliferation and reduced apoptosis (*59, 60*).

Dietary Factors Related to Decreased Colon Cancer Risk

Some dietary components have been suggested to lower the risk for colon cancer, e.g. a balanced diet with low amount of saturated fat and high amount of whole grains (*3*), fruits and vegetables (*4, 5*), as well as dietary fiber (*61*); habit of drinking tea (*62, 63*); frequent consumption of soybeans and soy food products (*64*); regular consumption of fish rich in n-3 polyunsaturated fatty acids (*65, 66*), etc. The underlined mechanisms for the protective effects can be roughly divided into five main categories (*67, 68*):

1) Interaction with and/or scavenging of carcinogenic substances exposed to colon epithelium, by compounds such as antioxidants and complex carbohydrates;

2) Breaking the unbalanced proliferation/apoptosis in malignant cells by induction of apoptosis and/or arrest of cell cycles, by compounds such as genistein, one of the isoflavones in soybeans (*69*) and many other polyphenols, n-3 polyunsaturated fatty acids, butyrate produced from dietary fiber, among others.;

3) Activation of cellular detoxifying/damage repairing/defensive mechanism, e.g. transcription factor Nrf2 (nuclear factor (erythroid-derived 2)-like 2) and associated transcriptional control element ARE (antioxidant response element);

4) Reduction of inflammation and suppression of related genes/molecular pathways, e.g. NF-κB ; and,

5) Suppression of oncogenes and restoration/maintaining of suppressed tumor suppressor genes.

It is noteworthy that many chemopreventive dietary components function through more than one of these mechanisms, which all work together to protect against colon tumor initiation and progression.

Sorghum in Cancer Prevention

Sorghum (*Sorghum bicolor*) is the fifth most important cereal crop in the world in terms of production (*23*). The agronomic advantages of sorghum (mainly drought and heat tolerance) make it suitable to grow in most arid and semi-arid areas around the world. Sorghum grain is a staple food and important cereal in many parts of Africa, Asia, and the Middle East (*16*). Sorghum in these areas is mainly consumed in the forms of couscous, porridge, baked goods (bread, flat bread, cookie, and steam bread), and fermented drinks. Sorghum is an important alternative grain to use in gluten-free diets (*70*). Besides grains, leaf sheaths of some red plant sorghum are used as food colorants in traditional African cooking (*71*). Sorghum is fairly resistant to pests and diseases, which is partly linked to its unique profile of phytochemicals (*72*).

In Africa and parts of Asia, regular consumption of sorghum and millets was associated with lower esophageal cancer incidences compared to areas where wheat and corn were the major cereals consumed (*73, 74*). In addition the amount of sorghum and millets consumed correlated with decreased incidence of esophageal cancer (*17*). On the other hand, increased incidence of esophageal cancer among South African blacks was

suggested to be due to substitution of sorghum with corn as a staple diet (*75*). These observations provided evidence that sorghum may have chemopreventive properties that other grains do not possess.

Black and tannin sorghum bran (6% in diet) decreased the number of azoxymethane induced premalignant lesions in the colons of rats compared with control diet (6% cellulose) (*18*). The sorghum brans lowered proliferation and induced apoptosis in colonocytes, which was possibly due to their ability to induce murine endogenous antioxidant enzyme activities (superoxide dismutase, catalase, and glutathione peroxidase) (*18*).

Hwanggeumchal (which means "golden" in color and "sticky" in texture) sorghum extracts reduced the growth of human breast tumor xenografts in mice and blocked metastasis to the lungs (*76*). The mechanism was linked to cycline D1 induced G1 cell cycle arrest and suppressed tumor growth by down regulation of Jak2/STAT pathways (*76*). Moreover this sorghum extract possessed higher capacity to decrease expression of oncogenic proteins than extracts from other grains, i.e. wheat and millet (*76*). However, the composition of the sorghum extract was not provided. A procyanidin-rich sorghum extract inhibited tumor growth in C57BL/6J mice bearing Lewis lung cancer by suppressing vascular endothelial growth factor (VEGF); in addition the sorghum extract improved the activity of antioxidant enzymes (superoxide dismutase and glutathione peroxidase) after D-galactose induced oxidative stress (*77*). These studies show that sorghum phenolics can suppress tumor progression in mouse models; however, the active components have not been identified.

We previously demonstrated that aqueous acetone extracts from sorghum grains of various phenolic profiles inhibited human esophageal (OE33) and colon adenocarcinoma (HT-29) cell growth *in vitro*, with IC_{50}s of 49.7-883 μg/mL. The antiproliferative capacity was correlated with their antioxidant efficacy, with tannin sorghum extracts as the most potent inhibitors followed by black sorghum rich in 3-deoxyanthocyanin pigments (*19*). In addition, we showed that specific non-tannin sorghum extracts induced the activity of quinone reductase (a phase II protective enzyme) in murine liver cells (Hepa1c1c7), with black sorghum as the most potent inducer. Further research with pure 3-deoxyanthocyanidins revealed a structure-activity relationship: *O*-methyl substitution on the A-ring increased the antiproliferative and QR inducing capacity of 3-deoxyanthocyanidins (*78*). This evidence indicated that phenolic composition of sorghum affects their chemopreventive potential (*19*).

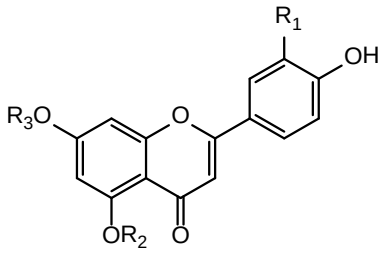
Phenolic Composition of Sorghum Grains

The composition of phenolic compounds in sorghum grains is influenced by genotypes (*24, 25*). Genetic factors control the presence of pigmented testa in sorghum grains, pericarp color of the grains, thickness of pericarp, and secondary plant color of sorghum (*79*). Sorghum grains with a pigmented testa under the pericarp accumulate high amounts of condensed tannins (proanthocyanidins), which are high-molecular weight polymerized flavan-3-ol and/or flavan-3,4-diol units (Figure 3) (*80*). Based on pericarp color, non-tannin containing sorghum grains can be divided into 3 main categories: white grains, with the least amount of phenolics, red (including the black)

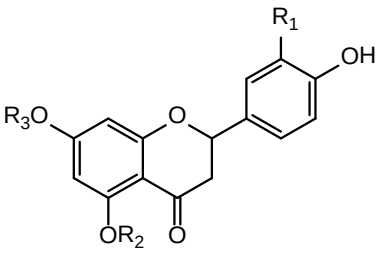
grains, with the highest amount of 3-deoxyanthocyanins (Table 1), as well as lemon-yellow grains, with the highest amounts of flavanones (Table 1) (*24, 25*). Sorghum secondary plant colors (i.e. tan, red and purple) also influence the phenolic composition of sorghum grains (Table 1). Sorghum grains with tan secondary plant color contain higher amounts of flavones than red/purple plants, while grains with red/purple plant color have higher 3-deoxyanthocyanins than the tan plants. On the other hand, the levels of flavanones are not affected by secondary plant color (*24, 25*). All sorghum grains contain phenolic acids, mainly hydroxycinnamic acids and their derivatives (*81, 82*).

Table 1. Levels (µg/g of ground sorghum grains, fresh weight) of flavonoids in different types of sorghum grains (*24, 25*).

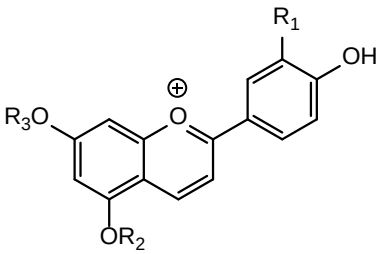
Criteria	Grain types	Compounds	Amount (µg/g)
Pericarp color	White	Flavones	19.4-65.0
		Flavanones	Not detected
		3-Deoxyanthocyanins	0.6-1.5
	Red (including Black)	Flavones	3.5-386
		Flavanones	8.1-241
		3-Deoxyanthocyanins	0.4-680
	Lemon-yellow	Flavones	20.5-362
		Flavanones	134-1780
		3-Deoxyanthocyanins	0.6-108
Secondary plant color	Tan	Flavones	19.0-386
		Flavanones	0-1428
		3-Deoxyanthocyanins	0-1
	Red/Purple	Flavones	0-67.0
		Flavanones	0-1780
		3-Deoxyanthocyanins	8.0-680



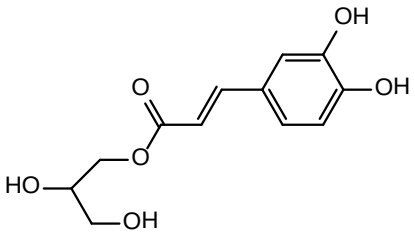
Flavones
R_1 = H: apigenin derivatives
R_1 = OH: luteolin derivatives



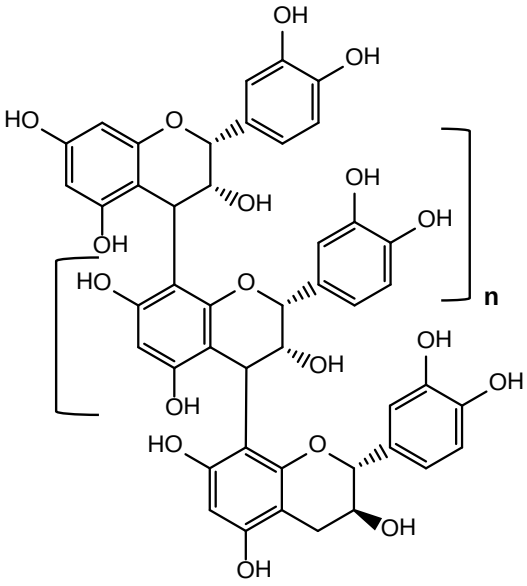
Flavanones
R_1 = H: naringenin derivatives
R_1 = OH: eriodictyol derivatives



3-Deoxyanthocyanins
R_1 = H: apigeninidin derivatives
R_1 = OH: luteolinidin derivatives



1-*O*-Caffeoylglycerol
(phenolic acid ester of glycerol)



Condensed tannins (proanthocyanidins)

Figure 3. Skeletal structure of major sorghum phenolics: 3-deoxyanthocyanins, flavones, flavanones, phenolic acid esters of glycerol and condensed tannins.

Sorghum grains have higher contents of polyphenols and antioxidant capacity than other major cereal grains, such as wheat, barley, millet and rye (*83*). Sorghum also contains phenolic compounds not commonly found in other cereal grains (Table 2). For example, flavanones (eriodictyol, naringenin and glycosides) are mainly present in citrus fruits (e.g. grapefruits, lemons, limes and oranges) (*84*) and flavones (derivatives of luteolin and apigenin) are mainly found in herbs and spices (e.g. parsley, dried oregano, and celery seeds) (*84*). The flavanol derivatives in sorghum are mostly of the condensed type (proanthocyanidins with degree of polymerization >10); barley and red finger millets are the other cereal grains with these compounds (*85*). Additionally, the extractable phenolic acids found in sorghum are mostly esters of glycerol (Figure 3) (*82*), which are different from the most commonly reported simple phenolic acids in grains. The pigments of black pericarp sorghums, 3-deoxyanthocyanins (Figure 3), are unique compared to anthocyanins, and sorghum is the only known edible source of such pigments. The 3-deoxyanthocyanins lack a substitution at the C-3 position of the C-ring, which makes them more stable as colorants (*29, 86*), and more cytotoxic than their anthocyanin analogs (*30*).

Table 2. Contents of major phenolic compounds in sorghum grains compared with other sources.

Compounds	Resources	Amount (μg/g)	References
Phenolic acids	Sorghum	385-746	(*87*)
	Barley	450-1346	(*88*)
	Millets (Finger, Foxtail and Pearl millets)	612-3907	(*89*)
	Corn (Maize)	601	(*88*)
	Oat	472	(*88*)
	Rice	197-376	(*88*)
	Rye	1362-1366	(*88*)
	Wheat	1342	(*88*)
Condensed tannins	Sorghum	7880-21970	(*90*, *91*)
	Barley	740	(*90*)
Anthocyanins	Red/Black pericarp Sorghum*	0.4-680	(*25*)
	Blue barley	4	(*92*)
	Pigmented corn	93-965	(*92*)
	Black rice	2283	(*92*)
	Blue/Purple wheat	13-153	(*92*)
Flavones	Sorghum	3.5-386	(*24*, *25*)
	Wheat	Not quantified	(*93*, *94*)
	Millets (Kodo, Finger, Foxtail, Proso, Pearl, and Little millets)	1.9-123.51	(*95*)
	Oats	Not quantified	(*96*)
	Parsley, dried	17746-135062	(*84*)
	Oregano, dried	9168-11465	(*84*)
	Celery seeds	8951	(*84*)
Flavanones	Sorghum	134-1380	(*24*, *25*)
	Citrus fruits (grapefruits, oranges, and lemons)	153.9-925.1	(*84*)

*Sorghum contains 3-deoxyanthocyanins (apigeninidin, luteolinidin and their derivatives)

As discussed earlier, observational studies, animal, and cell culture studies suggest directly or indirectly that phenolics present in sorghum grains contribute to chemoprevention. Possible mechanisms involved include antioxidant activity which is exerted by all phenolics, antiproliferation (induction of apoptosis or cell cycle arrest),

and anti-inflammation activity by specific polyphenols in sorghum. Many cellular signaling pathways have been associated with the chemopreventive benefits of phenolic compounds, such as activation of Nrf2 and suppression of NF-κB (*68*). Besides these, inherent estrogenic activity of the phenolic compounds may be a distinct chemopreventive mechanism, because of the potentially protective role of estrogen in gastrointestinal tract cancers. Among sorghum polyphenols, flavones and flavanones have been recognized as estrogenic (*31*). Based on structural similarities, 3-deoxyanthocyanins may possess estrogenic activity as well. Hence investigating estrogenic mechanism may provide valuable information on the role of sorghum polyphenols in cancer prevention.

Estrogen and Colon Cancer Prevention

Estrogen Signaling and Estrogen Receptors

The female steroid hormone, estrogen, is essential for the development and functions of female reproductive system and related mammary glands. Estrogen also plays an important role in other endocrine responsive systems, such as brain, bone, cardiovascular system and adipose tissue (*97*). Estrogen is believed to participate in the development and progression of various diseases, for example, cancer (breast, ovarian, colorectal, prostate), osteoporosis, cardiovascular diseases, and cognition diseases (*98*). The function of estrogen is mainly mediated through estrogen receptor (ER).

The estrogen receptor is a ligand-binding inducible transcription factor which after activation regulates the expression of target genes (*97*). Two ER subtypes have been discovered in humans and rodents: ERα and ERβ. The two ERs are ubiquitously expressed in many tissues but have distinct tissue distribution patterns and physiological functions. ERα is the major ER in reproductive systems, i.e. endometrium, breast, ovarian stroma, and hypothalamic tissue; while ERβ is the major ER in all other non-reproductive system related tissues, e.g. kidney, brain, bone, heart, lungs, intestinal mucosa, and endothelial cells (*98*).

The genomic (classical) action of estrogen is through ligand binding. Estrogen binds to the ligand binding domain of ER, then induces the ligand-specific conformational changes of the protein (so called "activation" of ER) (*99*). The ligand bound receptor (estrogen-ER) then dimerizes and binds to specific DNA-binding domain, estrogen responsive element (ERE), located within the promoter region of target genes. The estrogen-ER binding to ERE results in recruitment of coregulatory proteins (coactivators or corepressors) to the promoter, increase or decrease in mRNA levels and associated protein production, and finally, a physiological response (*98*).

The two ERs lead to different signaling pathways. Cell growth for ERα and apoptosis for ERβ (*100*). ERα is the main ER mediating the essential function of estrogen in the development of female reproductive systems. On the other hand, ERβ is believed to be a target of disease prevention/protection in tissues where it is predominantly expressed, such as the colon (*101*).

Mechanism of Estrogen and ERβ in Colon Cancer Prevention

Observational studies have found lower incidence of colon cancer in females compared with males (*8, 9*), and also in premenopausal women compared with post-menopausal women (*10*). Long term administration of oral contraceptives has been associated with reduced colon cancer incidence in women (*102*). Multiple observational studies showed approximately 30% reduction in relative risk for colon cancer in postmenopausal women who received hormone or estrogen replacement therapy compared with those who did not (*103-105*). These evidence suggest that estrogen plays a protective role in the development of colon cancer.

The role of ERβ in colon cancer prevention was discovered by the observed changes in expression pattern during colon cancer development. ERβ is the prevalent ER in normal colon mucosa and it is capable of modulating colonic physiological functions (*106*). However, its expression is progressively reduced during colon cancer development (*107-109*). In addition, the proliferative activity in colon adenomatous tissue was inversely correlated with the expression of ERβ (*109*). The major form of estrogen, 17β-estradiol (E_2), reduced non-malignant colonocytes growth *in vitro* and colonic tumor formation *in vivo* by ERβ mediated apoptosis (*11*) and *p53* activation (*12*). All these evidence suggest that ERβ is important in the early stage of colon carcinogenesis and could be a target in chemoprevention. However, the utilization of estrogen as a colon cancer chemopreventive agent remains controversial because elevated levels of estrogen are also associated with increased risk of breast cancer (*103-*

105). Selective ERβ agonists could thus be good alternatives for colon cancer chemoprevention.

Phytoestrogens and Colon Cancer Prevention

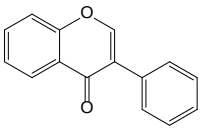
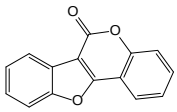
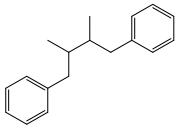
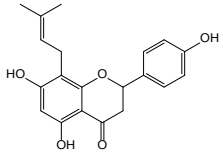
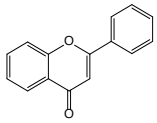
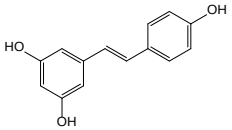
Characteristics of Phytoestrogens

Due to the ligand binding activation mechanism of ER, estrogens are not the only group of compounds which can bind to ER. Other compounds capable of binding to ER are called selective estrogen receptor modulators (SERMs), to which phytoestrogens belong. Phytoestrogens are a group of plant derived polyphenolic compounds which can modulate the activation of estrogen receptors, i.e. possess similar function as E_2 (*110, 111*). Steroid-like plant hormones are not considered as phytoestrogens because of their similar structure to the hormones in animals (*15*). Estrogenic compounds have been documented in more than 300 plant species, but only a few of these are found in the diets of human and animals. Among phytoestrogens, isoflavones from soybeans and lignans from flaxseed as well as whole grains are the two most common groups (*15*). The major groups of phytoestrogens and their origins are summarized in Table 3.

Phytoestrogens are more selective than estradiol in binding to ERβ and produce different estrogenic potencies after binding to ERs (*14, 112*). Genistein, for example, has a selective affinity to ERβ that is 7- to 48-fold higher than affinity to ERα (*113-115*). Genistein also produces different relative estrogenic potency compared to estradiol. When bound to ERα, genistein produces less than 1% of the activity of estradiol;

however, when bound to ERβ, it can produce up to 30-fold higher activity, depending on the assay system (*113-115*). The selective binding and higher potency of phytoestrogens towards ERβ make them good candidates for preventing cancers involving ERβ, such as colon cancer, esophageal cancer and other gastrointestinal tract cancers.

Table 3. Major phytoestrogens and their origin (*15, 116*).

Group name	Skeleton structure	Major compounds	Origins
Isoflavones		Genistein, daidzein, glycitein and their glycosides	Soybeans and other beans (*117*)
		BiochaninA	Chickpeas (*118*)
		Formononetin glycosides	Alfalfa sprouts (*119*)
Coumestans		Coumestrol	Clover, alfalfa sprouts (*119*), soybeans (*117*)
Lignans		Dimeric phenylpropanoids (mostly 8-8' linked): secoisolariciresinol and matairesinol	Flaxseeds, bran of most whole grains, and some fruits/vegetables (*120*)
Prenylflavonoids		Prenylnaringenin (especially 8-prenyl-)	Hops and beer (*121, 122*)
Others (weak/limited evidence)		Flavones	Spices (*123*)
		Resveratrol	Grapes and wine (*124, 125*)

Phytoestrogens in Colon Cancer Prevention

Multiple epidemiological studies have suggested that frequent consumption of soy foods (e.g. ≥ 4 times/week) reduced the risk of colon cancer, by 10-53% (*6*). Interestingly, the meta-analysis of relevant studies showed an approximate 21% reduction of colon cancer risk in women but not in men who consume soy foods (*6*). On the other hand, studies with consumption of soy or phytoestrogens indicate no elevated breast cancer risk (*14*).

A case-control study conducted at Toronto found higher intake of dietary lignans from typical Western diet (> 0.255 mg/day) was associated with a significant 27% reduction in colorectal cancer risk, compared with lowest dietary lignans consumption (0-0.158 mg/day); the same study also observed a similar reduction in colorectal cancer risk in people who consumed higher amounts of dietary isoflavones (>1.097 vs. 0-0.289 mg/day) (*126*).

A recent case-cohort study in Danish men and women examined the plasma concentrations of enterolactone, the microbial metabolites of lignans, and observed that with each doubling in enterolactone concentration, the relative risk for colon cancer in women decreased by 24%; no protective effect was found in men (*127*).

Many studies have looked at the mechanism by which phytoestrogens contribute to colon cancer prevention. Genistein is the most extensively studied phytoestrogen and has been shown to possess antiproliferative capacity (cell cycle arrest, apoptotic activity and tyrosine kinase inhibitor activity), and capacity to reduce the number of colonic ACFs and tumor numbers in animals treated with carcinogens (*128*). Enterolactone has

also been demonstrated to have antiproliferation, apoptotic and phase II enzyme (NADPH: quinone reductase) inducing capacity in various human colon cancer cells (*129*). Purified lignans from whole grains (flaxseed and rye) reduced the formation of ACFs and colonic polyps in mice studies (*129*).

Some studies have linked the colon cancer prevention capacity of phytoestrogens with activities mediated by ERβ, the predominant ER in the colon. For example, dietary soy isoflavones supplementation (genistein level 250 mg/kg diet) reduced tumor incidence in wild type and ERα-knockout ovariectomized mice, suggesting protection was ERα independent (*130*). Soy isoflavones decreased azoxymethane induced colon tumor size and burden in rats and inhibited human colon cancer cells (DLD-1) growth by elevating expression of ERβ (*131*). Further research by this group confirmed the antiproliferative effect was due to a G2/M cell cycle arrest mediated by ERβ (*69*). Diet enriched with cumestrol (ERβ agonist with a 200-fold higher affinity than E_2) was capable of reducing polyp numbers and increasing enterocyte migration in ovariectomized *APC*$^{+/-}$ mice (*132*). All these studies indicate that the protective effect of phytoestrogens against colon cancer development is likely linked to ERβ. Hence the activities mediated through ERβ should be considered in studies targeting estrogenic phenolic compounds in colon cancer prevention.

Methods Used to Determine Estrogenic Activity

Many methods, both *in vitro* and *in vivo*, have been utilized to determine estrogenic activities of various compounds. The major methods include:

1) Rat/mouse uterotrophic assays. These assays evaluate the effect of administered compounds in supporting female animal reproductive system development. The outcomes measured are weights of uteri, vagina, cervix, and records of vaginal openings, etc. (*133-135*). This assay determines the activities associated with ERα.

2) ER dependent responses in cell lines. These assays employ immortalized and proliferative cells which express or are transfected with ER expression vector and determine the typical cellular effect after activation of ER, such as increased/decreased cell proliferation, and expression of related genes/proteins (*135-137*).

3) Induction of transcriptioanal activity by estrogen response element (ERE) reporter. In these assays, ERE is constructed in conjunction with a reporter gene, such as luciferase and green fluorescent protein genes, then transfected into cell lines or yeasts expressing ER (as is or via transgenic approaches). The quantities of signals produced during the assay then reflect the transcriptional activity after administration of test compounds. A housekeeping gene (such as β-galactosidase) is always co-transfected to normalize the results (*138, 139*).

4) ER binding affinity assay *in vitro*. This assay measures the binding or competitive binding of target compounds to ER. Estradiol (E_2) is used as the binding competitor. The binding affinity is always expressed as molar concentration of 50% competition and this concentration is typically compared with that of E_2 (*133, 137*).

Most of the time, the activities determined in each of these assays are specific to one subtype of ER. Multiple assays from each category are used complementarily for one target compound due to the complexity of cell physiology. Stable and strong ER

antagonists, such as ICI 182, 780, have high binding affinity to ER but do not activate estrogen induced transcriptional activities (*140*). Parallel administration of ER antagonists eliminates the binding of estrogenic compounds to ER, which is also used to confirm ER mediated activities.

Models Used to Study Chemopreventive Potential of Estrogenic Compounds against Colon Cancer

In vitro *Cell Culture Models*

Many cell models derived from human colon adenocarcinoma have been used to study the chemopreventive potential of various compounds. Among the common cell lines are HT-29, Caco-2, LoVo, and HCT116. These models represent a wide range of disease progression stages and genetic make-up; hence are valuable tools to test hypotheses regarding colon cancer. However, all these cells are models of tumor progression. Information derived from cell culture models on how phytoestrogens affect the initiation and promotion stages of colon cancer is scarce, partly because of the difficulties in establishing cell lines from normal colonic epithelium.

The recently developed young adult mouse colonocytes (YAMCs) cell line provides a suitable model for studying the effect of dietary compounds in prevention of colon cancer during early stages (*141*). The cells are non-malignant, well characterized, morphologically primitive epithelial cells with no evidence of differentiation. The cell line was established from epithelial mucosa of a transgenic mouse expressing

temperature sensitive SV40 large T antigen gene constitutively. At permissive conditions (33 °C and the presence of interferon-γ), SV40 large T proteins in the cells are active and bind to *p53*, thus facilitating immortalization of the cells, whereas in non-permissive conditions (37-39 °C without interferon-γ), the conformation of the protein changes so that it can not bind to *p53* or immortalize cells (*142, 143*). Under these non-permissive conditions the cells behave as slow-proliferating, differentiated colonic epithelial cells and undergo apoptosis if they achieve maximal confluence. Therefore, the conditions are optimized for cells to proliferate slowly for 24 h at 39 °C and then undergo cell death over 5–8 days, similar to the life cycle of normal colon epithelium (*141*). Studies administrating test compounds at non-permissive conditions to this cell model provide mechanistic information on colon cancer prevention that correlate with *in vivo* studies (*11, 144*).

In vivo *Ovariectomized Rodent Models*

In vitro cell culture studies have some inherent disadvantages in providing microenvironment during normal colon tumorigenesis and interventions that can be applied to humans. Therefore, controlled *in vivo* animal studies are necessary to translate the hypothesis of potential chemopreventive compounds and targets derived from cell culture models to the physiology of colon (*145*). Rodent models (rats and mice) injected with colon specific carcinogens (most commonly 1,2-dimethylhydrazine, DMH and azoxymethane, AOM) are the most widely used models to study the pathogenesis of sporadic (adenoma-carcinoma sequence) colorectal cancer (*146*). The advantages of

carcinogen-induced rodent model include high reproducibility, similar pathogenesis as human colorectal cancer, especially at early stages, and ease of adaptation to animals with various genetic backgrounds (*146*). Ovariectomized animals are used in estrogenic compound studies to remove the effect from endogenous estrogens.

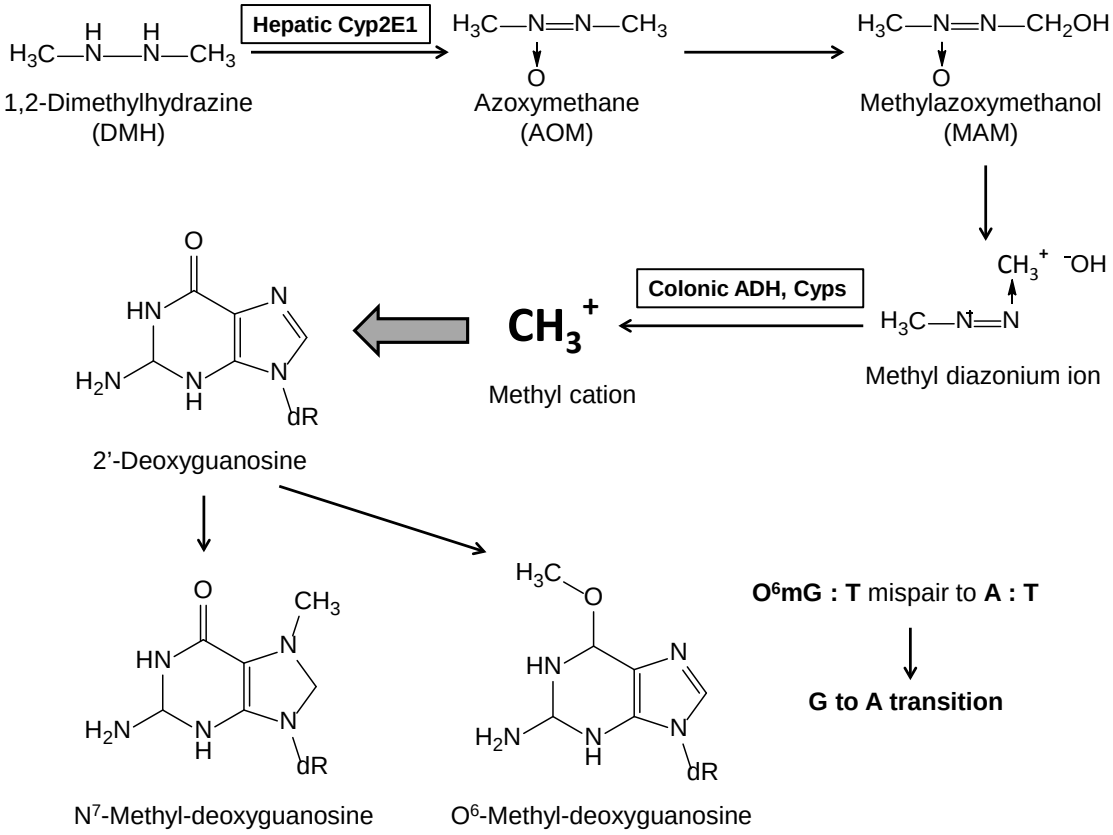


Figure 4. A scheme for the *in vivo* metabolism of colon carcinogens (1,2-dimethylhydrazine and azoxymethane) and corresponding G → A mutation of DNA. Figure adapted from Rosenberg et al. (*146*); Cyp2E1: a cytochrome P450 isoform; ADH: adenine dinucleotide^{+}-dependent dehydrogenase; Cyps: cytochromes P450.

Azoxymethane (AOM) is a colonic procarcinogen. The *in vivo* metabolized form of AOM is a DNA methylating agent, and guanine is its main target (*147*). Injection of AOM into rodent models induces formation of O^6-methylguanine adducts (*148*), which results in G → A mutations of DNA (Figure 4). This leads to formation of preneoplastic lesions, aberrant crypt foci (ACF), in the colon in a relatively short time (*43*). Pretlow et al. (*44*) observed ACFs in patients with colon cancer and suggested that these preneoplastic lesions were putative precursors of colon adenomas and carcinomas. Hence the preneoplastic lesion ACF induced by AOM has been used as a biomarker to evaluate the function and mechanisms of chemopreventive agents against colon cancer (*149, 150*).

In order to investigate estrogenic activity of sorghum phenolics and associated colon carcinogenesis protective mechanisms, *in vitro* cell models were first used to screen sorghum extracts of various phenolic profiles for estrogenic activity. For initial and primary screening to identify estrogenic potential of sorghum extracts, the human breast cancer cell line MCF-7 was used to identify estrogenic compounds. Thereafter, young adult mouse colonocytes (YAMCs) cells expressing primarily ERβ were used to investigate ERβ mediated activities induced by sorghum extracts in the early events of colon carcinogenesis. The major phenolic compounds in sorghum extracts were characterized and quantified by UPLC-MS and HPLC. The information on phenolic composition was used to identify possible estrogenic compounds in these extracts. With the information gathered from cell culture studies, specific sorghum extracts were tested

for their effects on mitigating the formation of ACFs in ovariectomized mice treated with AOM.

Effect of Thermal Processing on Sorghum Phenolics

Sorghum grains are typically thermally processed to make food for humans. The thermal processes include boiling, steaming, baking, flaking and extrusion, among the others. Structural changes induced by thermal processing can impact food quality and bioactive properties of polyphenols. Understanding the extent of degradation and degradation mechanisms of polyphenols would be helpful in predicting food product quality changes over time and exploring methods to stabilize the polyphenols during thermal processing.

Like other plant polyphenols, the amount and antioxidant capacity of sorghum phenolics are affected by heat treatments. After steam exposure at 220 °C for 120 s, extractable free phenolic acids in barley bran increase as high as 9-fold (*151*). Cooking (boiling water bath 12 min) whole sorghum meal (non-tannin local cultivar) increased free phenolic acids content, probably due to better extractability, which was accompanied by decrease of bound phenolic acids (*152*). Heat treatment increases hydrolysis of glycosidic bonds and ester/ether bonds between phenolic compounds, lignins, and hemicelluloses/cellulose, which results in the release of bound phenolic acids (*151*).

The antioxidant capacity of cooked sorghum meal reduced by roughly 50%, while the 3-deoxyanthocyanidin content reduced by 50-73% (*152*). The total phenols of

cooked sorghum grain meals was 38-65% lower compared to the uncooked meals (*153*). The antioxidant capacity of different types of sorghum (white, black and tannin) decreased 22-43% after baking in cookies and breads (*154*). Extrusion seems to decrease the antioxidants in sorghum the most: The total phenols of different varieties of sorghum grains and brans (white, black and tannin) reduced 59-87% after extrusion (*155*). Since degradation of polyphenols is temperature dependent, higher process temperature, such as frying and extrusion, results in higher loss of free phenolic compounds and antioxidant capacity. The decrease in antioxidant capacity could be because of heat induced structural degradation of phenolics (*156*).

The common reactions of flavonoids inducing by heat treatment include deglycosylation (*157*), break-down of ester bond, generation of C6-C3-C6 structure fragments (e.g. protocatechuic acid) (*158-160*), as well as formation of neo-formation compounds (e.g. hydroxymethylfurfural, melanoidins) (*161*). These reactions contribute to flavor and color development during cooking (*161*), but the degraded and/or oxidized compounds may be involved in unfavorable reactions, such as oxidation (*162*). Heat treatment depolymerizes proanthocyanidins into lower molecular weight units (*91*), which are known to be more bioavailable. Some polyphenols, e.g. anthocyanins, can polymerize during thermal processing, which are considered to retain more color during storage than the simple anthocyanins (*163, 164*).

Since thermal processing could affect the phenolic profiles and bioactivities of sorghum, such as estrogenic properties, it is important to consider the impact of this change on potential health benefits of sorghum phenolics. Among the sorghum

phenolics, the knowledge of thermal stability of 3-deoxyanthocyanins is very limited. These pigments possess unique characteristics as potential food colorants and chemopreventive agents. Therefore, determining their thermal stability would add valuable information on the potential of these pigments as bioactive food ingredients. In addition, knowing the degradation mechanisms and products is valuable in looking for strategies to stabilize the 3-deoxyanthocyanins during processing.

CHAPTER III

SORGHUM PHENOLIC EXTRACTS DEMONSTRATE ESTROGENIC ACTIVITY IN NON-MALIGNANT COLONOCYTES: RELATIONSHIP TO PHENOLIC PROFILES*

Introduction

Colon cancer is the third most prevalent type of cancer in the US, with 102,480 new cases and 50,830 deaths expected in 2013 (*1*). Colon cancer is also a common type of cancer worldwide, ranked as third most diagnosed cancer in males (663,600 new cases) and second in females (570,100 new cases), as well as the most commonly diagnosed malignancy in the digestive tract (*2*). Incidence of colon cancer in developing countries has increased significantly. Adaptation to Western diet is considered a likely contributor to this trend (*33, 34*). With increasing colon cancer medical expenditure on the patients (*49*) and public health sector (*50*), colon cancer is indeed a major public health burden in both developed and developing countries. Prospective studies suggest that as much as 70% of current colon cancer burden can be prevented by moderate modifications in dietary pattern and lifestyle (*51, 52*). Hence primary prevention seems to be the most effective strategy to "fight" colon cancer.

*Portions of this chapter were reprinted with permission from "Sorghum phenolics demonstrate estrogenic action and induce apoptosis in non-malignant colonocytes" by Yang, L., Allred, K. F., Geera, B., Allred, C. D., and Awika, J.M. Nutrition and Cancer **2012**, *64*, 419-427. Copyright 2012 by Taylor & Francis.

Consumption of whole grains has been associated with reduced risk of colon cancer (*3*). Besides the benefits from dietary fiber, minerals and vitamins, phenolic compounds present in whole grains are also important in preventing the onset of cancer, especially malignancies in the gastrointestinal tract. Consumption of sorghum was associated with lower incidence and mortality from esophageal cancer, a protection not associated with consumption of other cereal grains (*17, 73-75*). Sorghum bran and phenolic extracts inhibited colonic premalignant lesion formation (*18*) and breast/lung tumor progression in animal models (*76, 77*). Our previous studies showed that phenolic extracts of sorghum inhibited the growth of human esophageal (OE33) and colon (HT-29) adenocarcinoma cell growth *in vitro* at lower concentration than phenolic extracts from nuts (*20*) and grapes (*21*). However, the mechanisms involved and the active components are still unknown.

Of the many phenolic compounds in sorghum grains, flavones (e.g. apigenin and luteolin), flavanones (e.g. naringenin) and 3-deoxyanthocyanins are not present in large quantities in other cereal grains (*24, 25*). These compounds are known to contribute to chemoprevention via mechanisms other than antioxidant activity; for example, phase II enzyme inducing capacity and estrogenic activity. Estrogenic compounds are known to activate estrogen receptor-β (ERβ), the predominant estrogen receptor in the colon, thus in turn can induce apoptosis in damaged colonocytes, and activate tumor suppressor gene *p53 (11, 12)*, among other mechanisms. The aim of this study is to determine if inherent estrogen-like property of sorghum polyphenols could contribute to colon cancer

prevention. The hypothesis is that sorghum polyphenols possess estrogen-like properties and could consequently contribute to colon cancer prevention.

In this chapter, we first used *in vitro* cell models to screen for estrogenic potential of sorghum extracts of various phenolic profiles. The human breast cancer cell line MCF-7 is routinely used to identify estrogenic compounds; hence it was used for primary screening in this study. Thereafter, we used the young adult mouse colonocytes (YAMCs) expressing primarily ERβ to investigate ERβ mediated activities induced by sorghum extracts in the early events of colon carcinogenesis. The major phenolic compounds in sorghum extracts were characterized and quantified by UPLC-MS and HPLC. The information on phenolic composition was used to identify possible estrogenic compounds in these extracts.

Materials and Methods

Materials

Sorghum grains. Three varieties of sorghum with distinct differences in phenolic profiles were used: a white variety (ATX635×RTX436), a bright red variety (TX2911), and a black variety (TX430 black) (Table 4). The grains were kindly provided by Dr. W. L. Rooney in the Department of Soil & Crop Sciences, Texas A&M University. The White variety was harvested in 2001 and the others in 2008 in College Station, Texas. The seeds were stored at -20 °C till use.

Table 4. Characteristics of sorghum varieties used in this study (*26*).

Variety	Pericarp color	Secondary plant color	Major compounds
ATX635× RTX436	White	Tan	Phenolic acids, apigenin
TX2911	Bright red	Red	Phenolic acids, 3-deoxyanthocyanins, naringenin glycoside
TX430 (black)	Black	Purple	3-Deoxyanthocyanins, luteolin, apigenin, phenolic acids

Commercial standards of phenolic compounds. Pure standards were used to characterize and quantify phenolic compounds in sorghum. Apigenin and luteolin were from Indofine (Indofine Chemical Company, Inc., Hillsborough, NJ); eriodictyol and its 7-*O*-glucoside as well as apigenin- and luteolin-7-*O*-glucoside were from Extrasynthese (Extrasynthese, Genay Cedex, France); naringenin was from MP Biomedicals (Solon, Ohio); ferulic acid, caffeic acid, *p*-coumaric acid, and naringin were from Sigma-Aldrich (St. Louis, MO); 3-deoxyanthocyanidins, i.e. apigeninidin, luteolinidin, 7-*O*-methyl-apigeninidin, and 5-*O*-methyl-apigeninidin were from AlsaChim (Strasbourg, France).

Cell lines. MCF-7 cells were purchased from ATCC (Manassas, VA). The MCF-7 cells are estrogen-dependent tumor cells isolated from a postmenopausal woman with metastatic infiltrating ductal carcinomas and predominantly express ERα. Young Adult Mouse Colonocytes (YAMCs) cells were supplied by Dr. Robert Chapkin (Department of Nutrition and Food Science, Texas A&M University). These cells are a well-characterized non-malignant cell line derived from the Immortomouse, and are

morphologically primitive epithelial cells with no evidence of differentiation. The YAMC cells are conditionally immortalized by the expression of the temperature-sensitive SV40 large T antigen, and do not form tumors in athymic mice (*143*).

Methods

Extraction. Whole kernels of sorghum grain were ground in a cyclone mill (UDY, Boulder, CO) to pass through 0.1 mm screen before extraction. Ground samples were defatted using hexane at a ratio of 1:2 (w:v) by stirring for 2 h. The matrix was then centrifuged at 3100 ×g and the residue was dried inside a fume hood over-night at room temperature. Defatted samples were extracted with 70% (v/v) aqueous acetone with stirring for 30 min. Supernatant was collected by centrifuging (3100 ×g) for 15 min at 4 °C. Acetone was immediately removed from the supernatant under vacuum at 40 °C by a rotary evaporator (Rotovap, Büchi, Flawil, Switzerland). The aqueous phase was freeze-dried and used as a crude extract. Extracts were kept at -20°C till use.

Reconstitution of sorghum extracts. Freeze-dried sorghum grain extracts were reconstituted at 10 mg/mL with 100% methanol (HPLC grade, EMD Millipore, Billerica, MA) then diluted 5 times with 50% aqueous methanol for UPLC-MS identification and HPLC quantification.

UPLC-ESI-MS analysis. A Waters-ACQUITY UPLC/MS system (Waters Corp., Milford, MA) was used, which consisted of a binary solvent manager, autosampler (sample manager), column heater, and photodiode array eλ detector (PDA) and interfaced with a tandem quadrupole (TQD) mass spectrometer equipped with an ESI source. The separation was performed on a Kinetex C18 column (150 mm × 2.10 mm, 2.6 μm, Phenomenex, Torrance, CA) at 40 °C with following gradient at 0.4 mL/min: solvent A (0.05% formic acid in water), solvent B (acetonitrile), and the percentage of solvent B was 12-41% from 0-23.5 min, 41-75% from 23.5-25.5 min, 75% isocratic from 25.5-28.5 min, then 75-12% from 28.5-29.5 min, and 12% isocratic for 5 minutes to equilibrate the column. The injection volume was 1 μL. The monitoring wavelength for 3-deoxyanthocyanidins and derivatives was 485 nm; for phenolic acids and flavones was 340 nm; for flavanones and other polyphenols was 280 nm. Mass spectrometric data were acquired in positive mode for 3-deoxyanthocyanidins as well as its derivatives, and in negative mode for all the rest of compounds. Empower 2 software (Waters Corp.) was used to acquire and analyze data. The MS scan was recorded in the range of 100−1200 Da. Nitrogen was used both as a drying gas and as nebulizing gas, while argon was used as the collision gas (AOC, Bryan, TX). The nitrogen gas flow conditions were 800 and 50 L/h for desolvation and at the cone, respectively. The source block temperature and desolvation temperature were set at 150 and 400 °C, respectively. Optimization of ionization conditions was based on the intensity of the mass signals of protonated/deprotonated molecules and aglycones fragments and was performed for each individual peak/compound detected. Mass parameters were optimized as follows:

capillary voltage, 3.5/3.0 kV; and cone voltage, 60/30 V for positive/negative ionization, respectively. The MS/MS scan was optimized as follows: cone voltage of 60/(30−50) V and collision energy of (35-45)/(15−40) V. Compound identification was based on matching UPLC retention profile, UV-*vis* spectra and MS data with authentic standards. Where standards were not available, compounds were identified based on the fragment patterns compared with reports in the literature.

Quantitative analysis by HPLC. An Agilent 1200 series HPLC system (Agilent Technologies, Santa Clara, CA) was used for this analysis, which included a quadratic pump (with degaser), an autosampler, a column compartment, and a diode array detector (DAD). A Luna C-18 column (150 mm × 4.6 mm, 5.0 μm, Phenomenex, Torrance, CA) was used to carry out the separation with a two solvent gradient. Solvent A was 1% formic acid in water and solvent B was 1% formic acid in acetonitrile. The gradient based on solvent B was as follows: 0-3 min, 10%; 5 min, 18%; 10 min, 20%; 23 min, 26%; 25 min, 28%; 28 min, 40%; 30 min, 60%; 30-32 min, 60%; 34-40 min 10%. The column was kept at 40 °C during analysis and the flow rate was 1.0 mL/min. The injection volume was 10 μL. 3-Deoxyanthocyanins were quantified based on the signal at 480 nm, flavones and phenolic acids at 330 nm, and flavanones at 280 nm. The quantities of each compound were calculated based on standard curves obtained from pure compounds run under the same conditions.

Effect of sorghum extracts on MCF-7 cell growth. Potential of sorghum extracts to activate ERα was screened using an estrogen dependent human breast cancer cell line (MCF-7) expressing predominantly ERα. The MCF-7 cells were cultured in DMEM high glucose media (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS (Hyclone, Logan, UT) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA), at 37°C with 5% CO_2. The cells were sub-cultured every 7 days when 80-85% confluence was reached; media was changed every 2 or 3 days for the same passage of cells. The MCF-7 cells were cultured in DMEM media supplemented with charcoal-dextran treated FBS (CDFBS) for 48 hr prior to plating in order to eliminate the estrogen present in FBS supplemented media. DMEM media with CDFBS were used hereafter in the process. The MCF-7 cells were plated at 150,000 cells per well in 6-well plates. After incubating for 24 hr, cells were treated with sorghum extracts (White, TX2911 and TX430 (black)) at 1, 5, 10, 50, 100, 150 µg/mL. DMSO (0.1 %) in culture media served as the control in the dose-response curve. Estradiol (E_2, 1 nM) served as a positive control. Each dose for each extract was applied to 3 wells. The treatments in DMEM-CDFBS media were prepared by adding 1 µL of 1000x sorghum extract stock (reconstituted in DMSO) to 1 mL of DMEM-CDFBS media. Treatment media was changed after 48 hr to ensure the supply of treatment and essential nutrients. After 96 hr incubation with treatment, cells were trypsinized and harvested into microcentrifuge tubes. An aliquot of 20 µL of harvested cells was then added to 10 mL ISOTON II diluent (Beckman, Brea, CA) to determine cell number using a Z1 Coulter particle counter (> 4 µm, Beckman Coulter,

Brea, CA). The experiment was repeated on 3 different days; cell growth was reported by comparing the number of cells for each treatment with that of the control.

Estrogen response element-luciferase (ERE-luc) reporter assay. To confirm that the cell growth observed in MCF-7 cells was mediated by ER, estrogen response element-luciferase (ERE-luc) reporter assay was conducted. The MCF-7 cells were handled the same way as for determination of cell growth. Cells were plated at 80,000 cells per well in 24-well plates. After 24 hr, each well received in total 0.167 μg of ERE-luc plasmid DNA and 0.021 μg of β-galactosidase (β-GAL) plasmid DNA. The plasmid DNAs were distributed equally to each well with the aid of the transfection agent ESCORT (Sigma-Aldrich, St. Louis, MO). Transfection matrices were removed after incubating for 4 hr, and treatments (control, 0.1 % DMSO in culture media), positive control (1 nM estradiol), white sorghum (5, 10, 100 μg/mL), and TX430 (black) sorghum extracts (5, 10, 100 μg/mL) in DMEM-CDFBS media were added to cells (3 wells per treatment). Cells were further incubated for 18 hr; then lysed to determine the transcriptional activities by measuring firefly luciferase activity (luciferase assay kit, Promega E1500, Madison, WI) and constitutive β-galactosidase activity (β-galactosidase assay kit, Promega E2000, Madison, WI), according to the instructions of the manufacturer, by an Infinite M200 TECAN plate reader (Tecan Group, Männedorf, Switzerland). This experiment was performed in duplicates.

Effect of sorghum extracts on YAMC cell growth. Potential of sorghum extracts to activate ERβ was determined in a cell model predominantly expressing ERβ, non-malignant young adult mouse colonocytes (YAMCs). YAMC cells were cultured in RPMI 1640 media (Sigma-Aldrich, St. Louis, MO) supplemented with 5% FBS, 0.1% insulin, transferrin, selenious acid (ITS, BD Biosciences, Franklin Lakes, NJ), 1% Glutamax-1 (Invitrogen, Carlsbad, CA), and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). Cells were maintained under permissive conditions, 33˚C, with 10 units of interferon gamma (IFNγ) (Roche, Indianapolis, IN) per mL medium. Cells were cultured in CDFBS supplemented RPMI 1640 media 48 hr before plating and thereafter. Cells were plated at 25,000 cells per well in 6-well plates; then incubated at 33°C, without INFγ for 24 hr to allow attachment to the bottom of plate. Cells were then treated with control (0.1 % DMSO), positive control (1 nM estradiol), and treatments (sorghum extract, 1, 5, 10, 50 or 100 μg/mL) for 96 hr under non-permissive conditions (37 °C, without INFγ). Each treatment was applied to 3 wells. Treatment media was changed after the first 48 hr to ensure steady supply of treatments and nutrients. At the end of 96 hr incubation, cells were harvested with trypsin and counted with a Beckman Coulter particle counter (> 20 μm), as described earlier for MCF-7 cells. Relative growth of each treatment was calculated by comparing the cell numbers of each treatment with that of the control. Each experiment was repeated 3 times.

ER antagonist assay. In order to confirm the effect of sorghum extracts on YAMC cell growth was mediated through ER, ER antagonist ICI (ICI 182, 780; Fulvestrant, Sigma-

Aldrich, St. Louis, MO) was used. All the handling and plating procedures were the same as in determining YAMC cell growth. The only difference was in this set of experiments, each treatment was treated alone and co-treated with 1 μM ICI, each to 3 wells, respectively. Estradiol (1 nM) and estradiol co-treated with 1 μM ICI were used as the positive control and the concentration of DMSO in each treatment media was adjusted to 0.2%, as well as controls. Cell number and relative cell growth was determined as previously described using a Beckman Coulter particle counter. Each experiment was repeated 3 times.

Caspase-3 activity assay. In order to determine a possible mechanism for the growth inhibitory effect of sorghum extracts on YAMC cells, apoptosis was measured using a Caspase-3 Kit (EnzChek Caspase-3 Assay Kit No. 2, Invitrogen, Carlsbad, CA). The YAMC cells were cultured and treated the same way as in ER antagonist assay. After 96 hr of treatment, the cells were trypsinized and collected into microcentrifuge tubes. The cells were then lysed and the activity of caspase-3 was measured according to the instructions of the manufacturer. Relative apoptosis was determined by fluorescence intensity measured on a TECAN Infinite M200 plate reader. Three wells per treatment per experiment were used and four replicate experiments were conducted.

Identifying possible estrogenic compounds in sorghum extracts. From the results obtained from previous sorghum extracts and YAMC cell growth experiments, flavones and flavanones were identified as key components affecting estrogenic potential of

sorghum phenolic extracts. Hence two additional sorghum varieties with contrasting composition of flavones and flavanones were selected to test the hypothesis that flavones in sorghum are agonists while flavanones are antagonists of ERβ. The two new varieties were a dark red pericarp variety with tan secondary plant color (99LGWO50), which is high in flavones, mainly apigenin and luteolin; as well as a lemon-yellow pericarp variety with purple secondary plant color (SC748), which is high in flavanones, mainly glycosides of eriodictyol and naringenin (*26*). The sorghum grains were kindly provided by Dr. W. L. Rooney and Dr. Gary Peterson in the Department of Soil & Crop Sciences of Texas A&M University. The sorghum grains were harvested in 2008 at College Station, TX. The YAMC cells were used as a model in this set of experiments. ER antagonist assay was used to confirm the estrogenic effect. Pure commercially available apigenin and naringenin were used to further investigate relative contributions of flavones and flavanones to estrogenic properties of sorghum.

Hydrolysis of SC748 sorghum crude extract. From previous studies (*26*) we know that the flavanones in SC748 sorghum are mainly glycosides. In order to better evaluate the biological function of the flavanone aglycones, SC748 crude extract was hydrolyzed in acidified aqueous methanol (HCl : water : methanol = 0.1 : 49.9 : 50, v/v/v), at 60 °C for 27 hr in a water bath. Based on HPLC peak areas, 90% of the flavanone glycosides were hydrolyzed. Methanol was rotary evaporated and the aqueous fraction was freeze-dried. Total recovered solids after hydrolysis was 64.4% of that of before hydrolysis.

Statistical analysis. The data were analyzed with one way analysis of variance (ANOVA). Least square means were compared by Dunnett's t-test as a multiple comparison technique to determine the difference between treatments and their corresponding control. All statistical analysis was analyzed by SAS 9.2 (Cary, NC).

Results and Discussion

Characterization and Quantification of Major Phenolic Compounds in Sorghum Extracts

The White, red TX2911, and TX430 (black) sorghum varieties were initially selected to test their estrogenic activity based on the distinct differences in the compositions of 3-deoxyanthocyanins, phenolic acids, flavanones and flavones. The selection was based on our hypothesis that 3-deoxyanthocyanins, flavanones and flavones were estrogenic compounds in sorghum. Cell culture screening studies of these 3 sorghum varieties showed that White and TX430 (black) sorghum extracts possessed estrogenic activity while red TX2911 extract did not. The differences in flavones and flavanones seemed to be the most reasonable explanation for the differences in estrogenic activity. Hence two additional sorghum varieties were selected to confirm the roles of flavones and flavanones on estrogenic properties of sorghum. The two varieties were a dark red sorghum variety (99LGWO50) high in flavones and a lemon-yellow sorghum variety (SC748) high in flavanone glycosides (*26*). The phenolic composition of the 5 sorghum extracts were presented together in this session for simplicity.

Effect of hydrolysis on SC748 sorghum extract. SC748 sorghum extract contained glycosides of eriodictyol and naringenin as the major phenolic compounds (Figure 5). In order to better evaluate the estrogenic potential of flavanone aglycones, the SC748 extract was hydrolyzed in acidified methanol (HCl : water : methanol = 0.1 : 49.9 : 50, v/v/v) at 60 °C. After 27 hr of hydrolysis, more than 90% of the flavanone glycosides were hydrolyzed to corresponding aglycones, eriodictyol and naringenin (Figure 5). Hydrolysis also produced luteolin aglycone from its glycosides. Gallic acid (or an isomer) was also formed (Figure 5), probably as a degradation product of phenolic compounds due to heat and acid. Levels of other constituents, such as flavanone chalcones and phenolic acids, were not significantly affected (Figure 5). The hydrolyzed SC748 extract was denoted as SC748-H. In the hydrolyzed lemon-yellow sorghum extract SC748-H, the flavanone aglycones were the major phenolic constituents. The composition of SC748-H extract should fulfill our objectives in determining the role of flavanone aglycones on estrogenic property of sorghum.

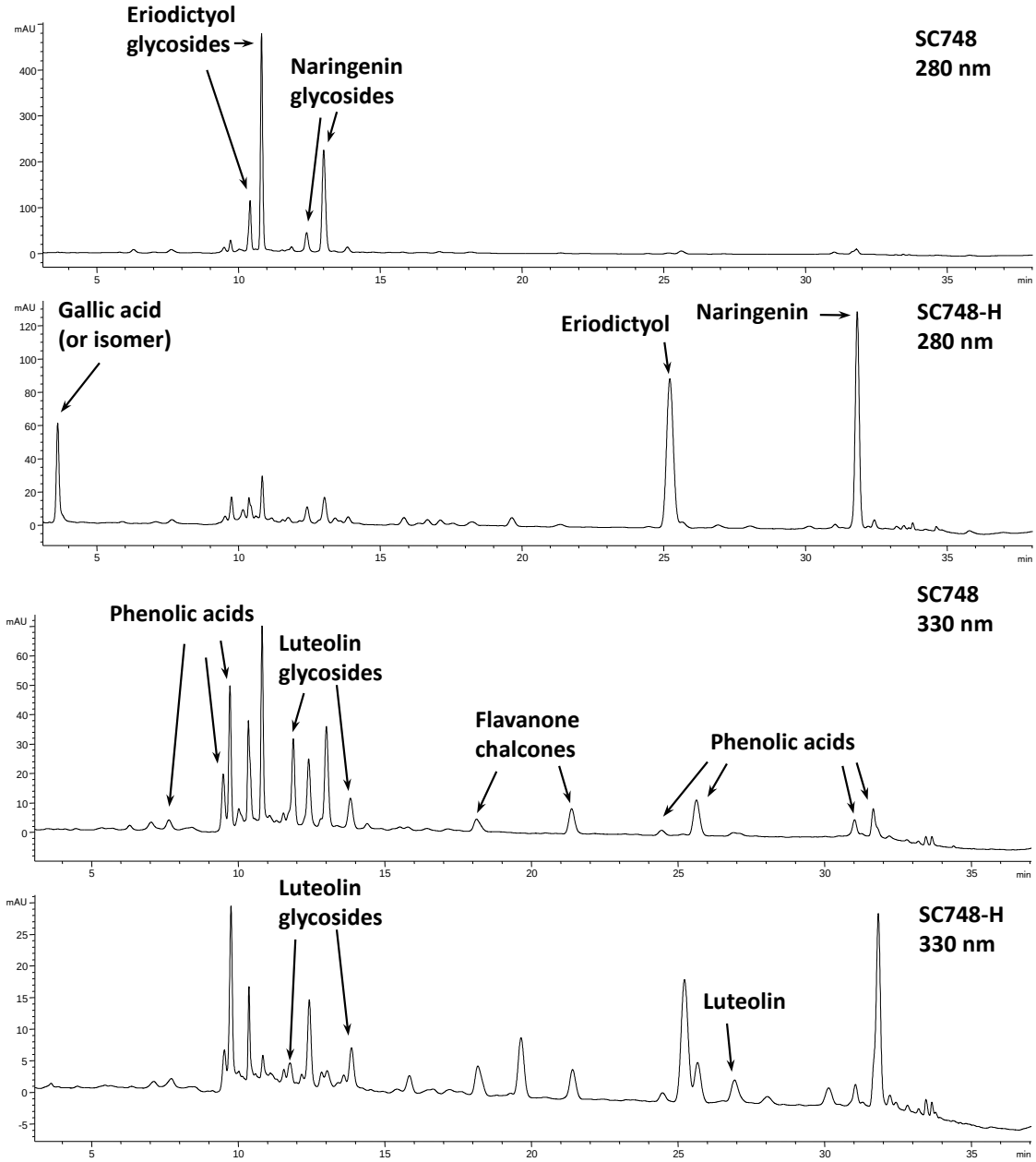


Figure 5. HPLC chromatograms of lemon-yellow sorghum extract before (SC748) and after acid hydrolysis (SC748-H) at 280 nm and 330 nm.

Phenolic acids and their esters. Phenolic acids, in their free and glycerol ester forms were detected in all sorghum extracts (Table 5, Figure 6). All were hydroxycinnamic acid derivatives, mainly caffeic acid and ferulic acid derivatives (Figure 7).

Peak 1 (t_R = 1.564 min, λ_{max} = 316 nm) had a $[M-H]^-$ at *m/z* 415. The MS/MS fragmentation pattern showed a dominant ion (100% intensity) at *m/z* 253 (M-162 amu, loss of a hexose unit). **Peak 2** (t_R = 1.749 min, λ_{max} = 325 nm) had the same $[M-H]^-$ at *m/z* 415. Besides the fragment ion of *m/z* 253, it also had ions of *m/z* 179 and *m/z* 161, which matched fragmentation pattern of caffeoylglycerol (Table 5). According to Ma et al. (*165*), caffeoyl moiety substitutes at the 2-*O*- position elutes earlier than the one at the 1-*O*- position. Thus, **peak 1** was identified as 2-*O*-caffeoylglycerol-*O*-glucoside and **peak 2** as 1-*O*-caffeoylglycerol-*O*-glucoside. These two isoforms of glucosylated caffeoylglycerol were only detected in White sorghum extract. The presence of caffeoylglycerol has been reported in red sorghum (*82*).

Peak 3 (t_R = 1.813 min, λ_{max} = 325 nm) had a $[M-H]^-$ at *m/z* 253. **Peak 4** (t_R = 2.199 min, λ_{max} = 325 nm) had the same $[M-H]^-$ at *m/z* 253. The MS/MS fragmentation pattern of these two compounds was the same: Both showed a dominant ion at *m/z* 161 and ions of *m/z* 179 and *m/z* 135, which matched fragmentation pattern of caffeoylglycerol (Table 5). Thus, **peak 3** was identified as 2-*O*-caffeoylglycerol and **peak 4** as 1-*O*-caffeoylglycerol. The 2-*O*-caffeoylglycerol was found in all sorghum extracts except SC748-H, and the 1-*O*-caffeoylglycerol was found in all sorghum extracts as a dominant peak. The presence of caffeoylglycerol has been reported in red sorghum (*82*).

Peak 5 (t_R = 2.286 min, λ_{max} = 325 nm) had a $[M-H]^-$ at *m/z* 179. The MS/MS showed only one dominant ion at *m/z* 135 (M-44 amu, loss of a carboxyl unit), which matched the pattern for caffeic acid (Table 5). Thus, **peak 5** was identified as caffeic acid. All 5 sorghum extracts contained caffeic acid, which was in accordance with previous reports (*24, 25*).

Table 5. Identification of phenolic acids and their esters in sorghum extracts (monitored at 340 nm) based on UPLC retention time (t_R), UV-*vis* spectroscopic characteristics (λ_{max}), and MS-MS/MS spectroscopic pattern. Ionization was performed in the negative mode. Peak numbers are referenced to Figure 6.

Peak No.	**t_R (min)**	**λ_{max} (nm)**	**$[M-H]^-$ (*m/z*)**	**MS/MS Fragments (*m/z*)**	**Proposed Identification**
1	1.564	316	415	253	2-*O*-Caffeoylglycerol-*O*-glucoside
2	1.749	325	415	253, 179, 161, 135, 119	1-*O*-Caffeoylglycerol-*O*-glucoside
3	1.813	325	253	179, 161, 135	2-*O*-Caffeoylglycerol
4	2.199	325	253	179, 161, 135	1-*O*-Caffeoylglycerol
5	2.286	325	179	135	Caffeic acid
6	3.735	294	355	193	Ferulic acid-*O*-galactoside or galactosyl ferulate
7	4.355	316	355	193	Ferulic acid-*O*-glucoside or glucosyl ferulate
8	6.709	325	193	161, 134	Ferulic acid
9	9.841	327	415	253, 179, 161, 135	Dicaffeoylglycerol
10	10.228	326	415	253, 235, 179, 161, 135	Dicaffeoylglycerol
11	10.703	322	415	253, 179, 161, 135	Dicaffeoylglycerol
12	12.568	315	399	253, 235, 179, 163, 161, 145	*p*-Coumaroyl-caffeoyl-glycerol
13	15.655	314	413	193, 177, 163, 145, 134, 119	*p*-Coumaroyl-feruloyl-glycerol
14	16.135	329	443	193, 175, 149, 134	Diferuloylglycerol
15a	13.075-a	296	399	253, 235, 163, 145, 119	*p*-Coumaroyl-caffeoyl-glycerol
15b	13.075-b	296	429	253, 249, 193, 179, 161, 134	Feruloyl-caffeoyl-glycerol

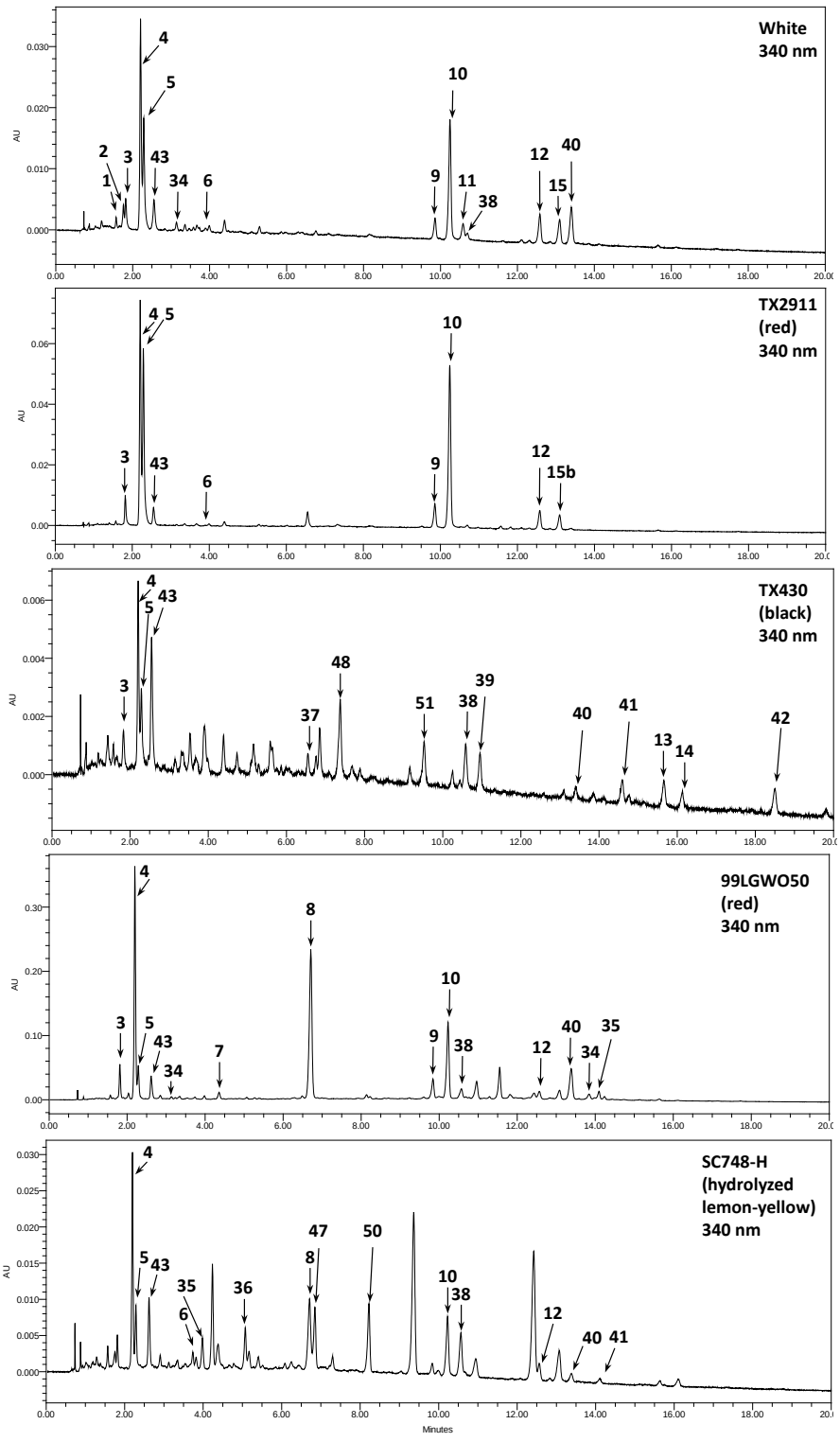


Figure 6. Reverse-phase UPLC chromatograms of sorghum extracts at 340 nm. Peak numbers are referenced to Tables 5 and 9.

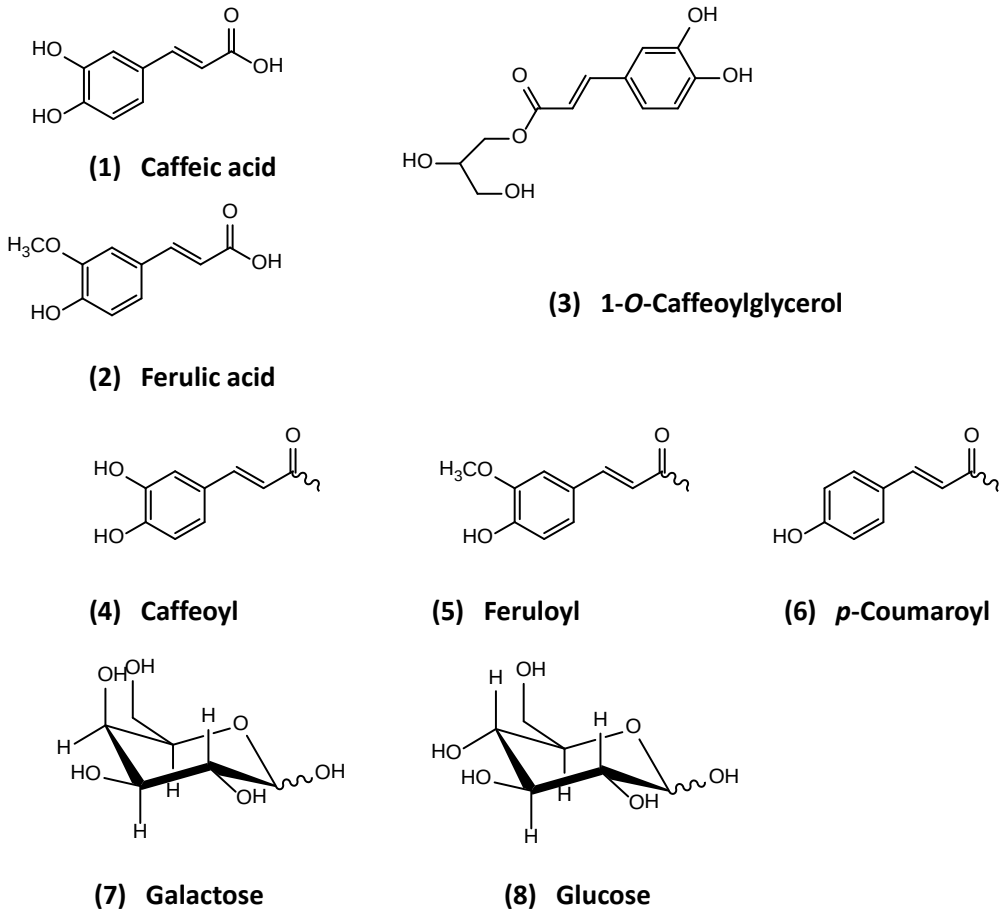


Figure 7. Chemical structures of phenolic acids and their glycosyl and glycerol ester dirivatives detected in sorghum.

Peak 6 (t_R = 3.735 min, λ_{max} = 294 nm) had a $[M-H]^-$ at *m/z* 355. **Peak 7** (t_R = 4.355 min, λ_{max} = 316 nm) had the same $[M-H]^-$ at *m/z* 355. The MS/MS of these two compounds included only one dominant ion at *m/z* 193 (M-162 amu, loss of a hexose unit), which matched fragmentation pattern of ferulic acid-*O*-glycoside or ferulic acid glycoside ester (Table 5). In reverse-phase HPLC, the eluting sequence of glycosides is reported to be galactoside, glucoside, then arabinoside (*166*). Thus, **peak 6** was

tentatively identified as ferulic acid-*O*-galactoside (or galactosyl ferulate) and **peak 7** as ferulic acid-*O*-glucoside (or glucosyl ferulate). **Peak 6** was identified in White, TX2911, and SC748-H extracts, and **peak 7** was only found in 99LGWO50 extracts.

Peak 8 (t_R = 6.709 min, λ_{max} = 325 nm) had a $[M-H]^-$ at *m/z* 193. The MS/MS fragments included ions at *m/z* 161 and at *m/z* 134 (dominant ion, M- 59 amu, loss of a C-COOH unit). Thus, **peak 8** was identified as ferulic acid (Table 5). Interestingly, the free form of ferulic acid was only detected in 99LGWO50 as a major peak, but not in other sorghum samples. The free form of ferulic acid was also detected in SC748 after hydrolysis (SC748-H). Glycosides (or esters) of ferulic acids were detected in each variety of sorghum, indicating most sorghum varieties contain the ester forms of phenolic acids.

Peak 9 (t_R = 9.841 min, λ_{max} = 327 nm), **Peak 10** (t_R = 10.228 min, λ_{max} = 326 nm), and **Peak 11** (t_R = 10.703 min, λ_{max} = 322 nm) all had a $[M-H]^-$ at *m/z* 415. The MS/MS fragmentation patterns of these three compounds were almost identical: ions at *m/z* 253 (M- 162 amu), *m/z* 179 (M- 162 amu – 74 amu) and *m/z* 135 (M- 162 amu – 74 amu – 44 amu), matched the fragments of caffeoylglycerol, caffeoyl, and caffeoyl unit -COOH, respectively (Table 5) (*165*). Thus, **peaks 9, 10, 11** were identified as dicaffeoylglycerols. The substitution pattern can be 1, 3-, 1, 2-, and 2, 3-dicaffeoylglycerol. **Peak 9** was detected in White, TX2911, and 99LGWO50 sorghum extracts. **Peak 10** was detected in White, TX2911, 99LGWO50, and SC748-H extracts. **Peak 11** was only detected in the White sorghum extract. The presence of dicaffeoylglycerol has been reported in red sorghum (*82*).

Peak 12 (t_R = 12.568 min, λ_{max} = 315 nm) had a $[M-H]^-$ at *m/z* 399. The MS/MS fragmentation pattern included ions at *m/z* 253, *m/z* 179 and *m/z* 163, matched characteristic fragments of caffeoylglycerol, caffeoyl, and coumaroyl units, respectively (Table 5). The ion corresponding to the backbone structure of a glycerol without two substituted –COO unit is C_3H_5O, which also matched the pattern of the fragments (M- 179 amu- 163 amu = 57 amu = C_3H_5O). Thus, **peak 12** was identified as *p*-coumaroyl-caffeoyl-glycerol. It was detected in all the sorghum extracts except TX430 (black) extract. The presence of *p*-coumaroyl-caffeoyl-glycerol was reported in red sorghum (*82*).

Peak 13 (t_R = 15.655 min, λ_{max} = 314 nm) had a $[M-H]^-$ at *m/z* 413. The MS/MS fragmentation pattern included ions at *m/z* 193 and *m/z* 163, which matched characteristic fragments of feruloyl and coumaroyl units, respectively (Table 5). The mass of this compound also matched the pattern of phenolic acid glycerol ester (M- 193 amu - 163 amu = 57 amu = C_3H_5O). Thus, **peak 13** was identified as *p*-coumaroyl-feruloyl-glycerol. It was only detected in TX430 (black) extract. The presence of *p*-coumaroyl-caffeoyl-glycerol was reported in red sorghum (*82*).

Peak 14 (t_R = 16.135 min, λ_{max} = 329 nm) had a $[M-H]^-$ at *m/z* 443. The MS/MS fragmentation pattern included one dominant ion at *m/z* 193, which matched characteristic fragments of a feruloyl unit (Table 5). Thus, **peak 14** was identified as diferuloylglycerol. It was only detected in TX430 (black) extract. The presence of diferuloylglycerol has not been reported in sorghum so far.

Peak 15 (t_R = 13.075 min, λ_{max} = 296 nm) was a mixture. In the White sorghum extract, this peak had a $[M-H]^-$ at *m/z* 399 (75% intensity) and a $[M-H]^-$ at *m/z* 429 (100% intensity); in the TX2911 extract, this peak was pure and had a $[M-H]^-$ at *m/z* 429. Hence we designated compound with *m/z* 399 as **peak 15a** and compound with *m/z* 429 as **peak 15b**. The MS/MS fragmentation pattern of *m/z* 399 was almost identical to that of **peak 12**, thus **peak 15a** was identified as *p*-coumaroyl-caffeoyl-glycerol (Table 5). It was only detected in the White sorghum extract. **Peak 15a** is an isomer of **peak 12**, the substitution pattern can be 1, 3- or 1, 2- *p*-coumaroyl-caffeoyl-glycerol. The MS/MS fragments of **peak 15b** included ions at *m/z* 253 and *m/z* 193, matched characteristic fragments of caffeoylglycerol and feruloyl units, respectively (Table 5). The mass of this compound also matched the pattern of phenolic acid glycerol ester (M-179 amu -193 amu = 57 amu = C_3H_5O). Thus, **peak 15b** was identified as feruloyl-caffeoyl-glycerol. The presence of feruloyl-caffeoyl-glycerol has not been reported in sorghum, but has been identified by Ma et al. in *Ananas comosus* L. leaves (*165*).

Quantification of phenolic acids and esters in sorghum extracts. The contents of phenolic acids and esters in 5 sorghum extracts are shown in Table 6. The bright red sorghum TX2911 extract had the highest amount of phenolic acid derivatives among the 5 sorghum extracts (119.7 mg/g extract) followed by the dark red sorghum 99LGWO50 extract (79.3 mg/g extract). The black sorghum TX430 (black) and the hydrolyzed lemon-yellow sorghum extract (SC748-H) contained the least amount of phenolic acid derivatives (4.25 and 5.10 mg/g extract, respectively).

Table 6. Contents (mg/g extract) of phenolic acids and their esters in sorghum extracts as quantified by HPLC-DAD system[a].

Compound	Peak No.	White	TX2911 (red)	TX430 (black)	99LGWO50 (red)	SC748-H (hydrolyzed lemon-yellow)
2-*O*-Caffeoyl-glycerol-*O*-glucoside[b]	1	Trace	ND	ND	ND	ND
1-*O*-Caffeoyl-glycerol-*O*-glucoside[b]	2	Trace	ND	ND	ND	ND
2-*O*-Caffeoyl-glycerol[b]	3	0.479 ± 0.08	2.25 ± 0.007	0.138 ± 0.01	1.85 ± 0.32	0.211 ± 0.014
1-*O*-Caffeoyl-glycerol[b]	4	3.60 ± 0.94	15.71 ± 0.004	1.54 ± 0.07	12.36 ± 2.04	1.33 ± 0.016
Caffeic acid	5	1.16 ± 0.26	7.65 ± 0.66	0.547 ± 0.03	6.86 ± 1.11	0.175 ± 0.006
Ferulic acid-*O*-galactoside[c]	6	0.594 ± 0.15	0.954 ± 0.04	ND	ND	0.334 ± 0.006
Ferulic acid-*O*-glucoside[c]	7	ND	ND	ND	0.746 ± 0.14	ND
Ferulic acid	8	ND	ND	ND	0.512 ± 0.08	0.378 ± 0.002
Dicaffeoyl-glycerol[b]	9	2.18 ± 0.93	10.2 ± 1.08	ND	8.15 ± 1.14	ND
Dicaffeoyl-glycerol[b]	10	10.97 ± 5.0	64.8 ± 6.77	ND	38.29 ± 5.15	1.48 ± 0.15
Dicaffeoyl-glycerol[b]	11	Trace	ND	ND	ND	ND
p-Coumaroyl-caffeoyl-glycerol[b,d]	12	4.76 ± 1.56	11.0 ± 1.39	ND	5.50 ± 0.22	1.19 ± 0.25
p-Coumaroyl-feruloyl-glycerol[c,d]	13	ND	ND	1.14 ± 0.06	ND	ND
Diferuloylglycerol[c]	14	ND	ND	0.880 ± 0.07	ND	ND
Feruloyl-caffeoyl-glycerol[b,c]	15b	3.05 ± 1.26	7.09 ± 0.86	ND	5.02 ± 0.66	ND
Total phenolic acids and esters		26.8 ± 10.2	119.7 ± 10.8	4.25 ± 0.02	79.3 ± 10.9	5.10 ± 0.08

[a]All values are expressed as mean ± SD of two separate runs. [b]Based on molar extinction coefficient of caffeic acid. [c]Based on molar extinction coefficient of ferulic acid. [d]Based on molar extinction coefficient of *p*-coumaric acid. ND = not detected. Peak numbers are referenced to Figure 6.

Despite the differences in quantities, composition of phenolic acid and their derivatives were generally similar across all samples. Caffeoylglycerols and dicaffeoylglycerols were the major phenolic acids identified. Two isomers of caffeoylglycerols were found in all sorghum extracts; while dicaffeoylglycerol isomers

were detected in all sorghum extracts except TX430 (black). On the other hand, the TX430 (black) extract contained two derivatives feruloyl-glycerol, *p*-coumaroyl-feruloyl-glycerol and diferuloylglycerol, which were not present in other sorghum extracts. Besides the common phenolic acid esters found in all sorghum extracts, White sorghum extract also contained two glucosides of caffeoylglycerol, and an additional dicaffeoylglycerol isomer, though in trace amounts.

Free phenolic acids were previously assumed to be the major constituents of readily extractable phenolic acids in sorghum, which resulted in very low quantitative data (*87*). Recently, Svensson et al. (*82*) reported that most extractable phenolic acids in a red pericarp sorghum were actually glycerol esters. Our data confirm this finding, and demonstrate significant variation in types and quantities of the phenolic acid esters in different sorghum varieties.

Flavanones. Flavanones were identified mainly as eriodictyol and naringenin derivatives (Table 7, Figure 8).

Peak 16 (t_R = 2.598 min, λ_{max} = 285 nm) had a $[M-H]^-$ at *m/z* 449. **Peak 17** (t_R = 2.905 min, λ_{max} = 283 nm) had the same $[M-H]^-$ at *m/z* 449. The MS/MS of these two compounds included a dominant ion at *m/z* 287 (M- 162 amu, loss of a hexose unit), which matches fragmentation pattern of eriodictyol glycoside (Table 7) (*82*). In reverse-phase HPLC, galactosides elute sooner than glucosides and arabinosides (*166*), the retention time of eriodictyol-7-*O*-glucoside standard was at 4.434 min, which was later than the two glycosides detected. Thus we inferred these two compounds were

galactosides of eriodictyol, but substituted at different positions. Though 7-*O*-glycosides are more common, both 5-*O*- and 7-*O*-glucosides of eriodictyol have been reported in sorghum (*82, 167*). According to Chang et al., glycosides substituted at the C-7 position increases polarity hence elutes earlier than those substituted at the C-5 position (*168*). Thus, **peak 16** was tentatively identified as eriodictyol-7-*O*-galactoside and **peak 17** as eriodictyol-5-*O*-galactoside. Both of these two compounds were identified in the lemon-yellow sorghum extracts only, both SC748 and hydrolyzed SC748-H extracts.

Peak 19 (t_R = 3.982 min, λ_{max} = 289 nm) and **Peak 20** (t_R = 4.385 min, λ_{max} = 283 nm) had the same $[M-H]^-$ at *m/z* 433. The MS/MS of these two compounds included a dominant ion at *m/z* 271 (M- 162 amu, loss of a hexose unit) and a diagnostic ion at *m/z* 151, which matched fragmentation pattern of naringenin glycoside (Table 7) (*82*). The retention time of eriodictyol-7-*O*-glucoside standard was at 4.43 min, and naringin (naringenin-7-*O*-rutinoside) standard was at 6.28 min. Naringenin mono-glucosides should elute in between of these two compounds. Generally, galactosides elute sooner than glucosides, which elute earlier than rutinosides. Thus we inferred the two naringenin isomers were galactosides of naringenin substituted at different positions. According to the eluting sequence of glycosides substituted at different A-ring positions (*168*), **peak 19** was tentatively identified as naringenin-7-*O*-galactoside and **peak 20** as naringenin-5-*O*-galactoside. Naringenin-7-*O*-glucoside has been identified in red sorghum (*82*). **Peak 19** and **peak 20** were detected in SC748-H extracts; whereas **peak 20** was also detected in TX430 (black), TX2911, and 99LGWO50 extracts (as minor peaks). Flavanones are intermediates of 3-deoxyanthocyanin biosynthesis (*169*), thus the

presence of naringenin galactoside in TX430 (black) and the two red sorghum extracts (TX2911 and 99LGWO50) may be related to 3-deoxyanthocyanins present in these extracts.

Table 7. Identification of flavanones and their derivatives in sorghum extracts (monitored at 280 nm) based on UPLC retention time (t_R), UV-*vis* spectroscopic characteristics (λ_{max}), and MS-MS/MS spectroscopic pattern. Ionization was performed in the negative mode. Peak numbers are referenced to Figure 8.

Peak No.	**t_R (min)**	**λ_{max} (nm)**	**$[M-H]^-$ (*m/z*)**	**MS/MS fragments (*m/z*)**	**Proposed Identification**
16	2.598	285	449	287	Eriodictyol-7-*O*-galactoside
17	2.905	283	449	287, 151, 135	Eriodictyol-5-*O*-galactoside
18	3.535	284	883	721, 595, 541, 433, 405, 297, 287	Pyrano-3', 4', 5', 5, 7-pentahydroxyflavanone-(3 → 4)-catechin-7-*O*-glucoside
19	3.982	289	433	271, 151	Naringenin-7-*O*-galactoside
20	4.385	283	433	271, 151	Naringenin-5-*O*-galactoside
21	4.735	284	867	705, 579, 551, 525, 431, 417, 389, 271	Pyrano-eriodictyol-(3 → 4)-catechin-7-*O*-glucoside
22	4.884	278	449	287, 193, 147, 139	Unknown
23	6.552	283	851	689, 563, 551, 401, 389, 125	Pyrano-naringenin-(3 → 4)-catechin-7-*O*-glucoside
24	9.365	287	287	151, 135, 107	Eriodictyol
25	10.227	279	287	193, 191, 151, 147, 139, 135	Unknown
26	10.962	295	689	509, 401, 389, 281, 271, 255, 125	Pyrano-naringenin-(3 → 4)-catechin
27	11.552	295	689	509, 401, 389, 281, 271, 255, 125	Pyrano-naringenin-(3 → 4)-catechin
28	11.813	295	689	509, 401, 389, 271, 255, 125	Pyrano-naringenin-(3 → 4)-catechin
29	11.821	295	1107	705, 673, 417, 401, 271	Pyrano-naringenin-(3 → 7)-pyrano-eriodictyol-(3 → 4)-catechin
30	12.422	289	271	177, 151, 119, 107	Naringenin
31	13.073	326	301	286, 177, 164, 151, 149, 123, 107	3'-*O*-methyl-Eriodictyol or 4'-*O*-methyl-Eriodictyol
32	13.843	302	773	473, 441, 401, 209	Pyrano-3-deoxyflavan-3-ol-(3 → 3)-pyrano-naringenin
33	14.097	302	773	473, 401, 209	Pyrano-3-deoxyflavan-3-ol-(3 → 3)-pyrano-naringenin

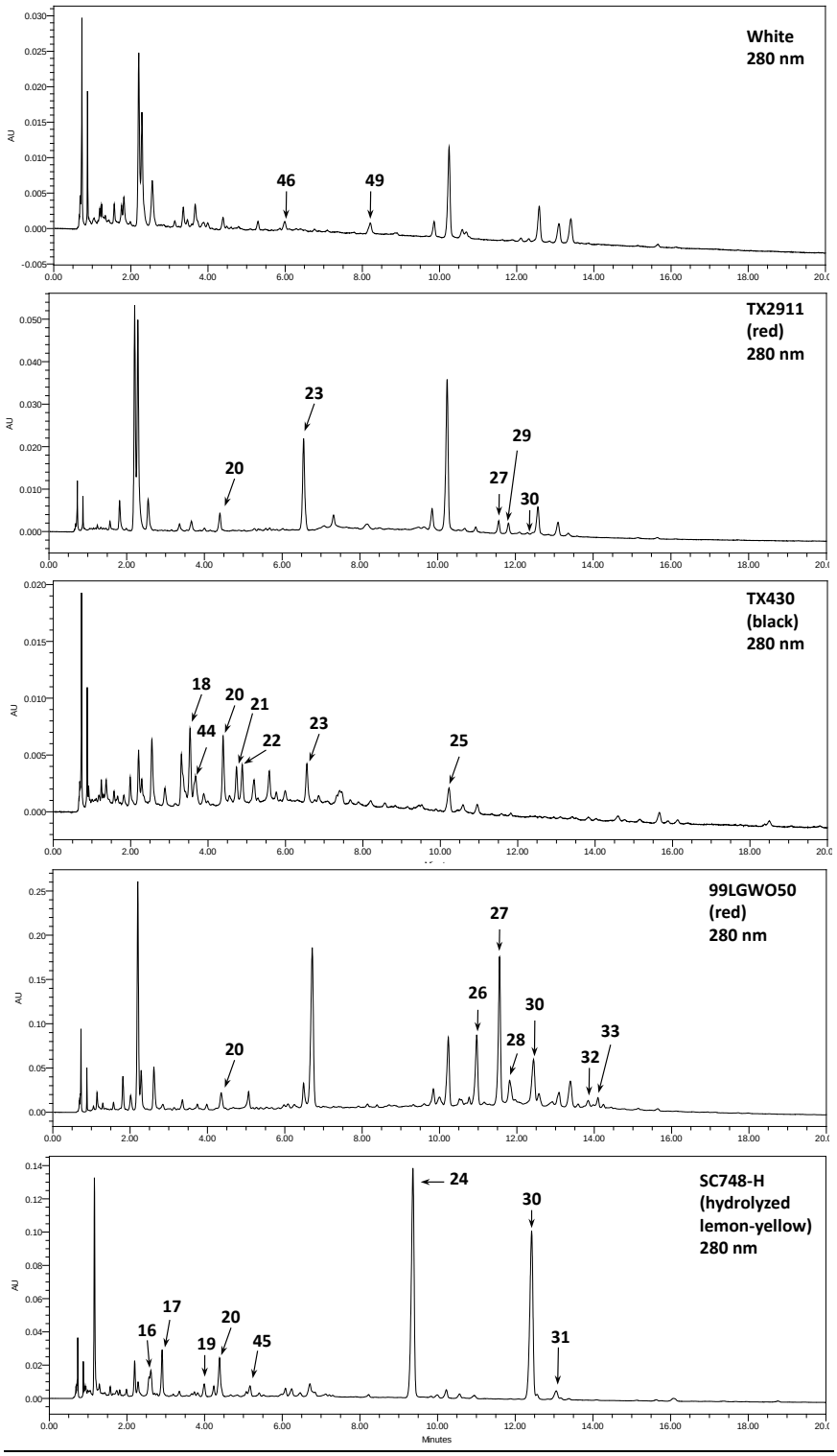


Figure 8. Reverse-phase UPLC chromatograms of sorghum extracts at 280 nm. Peak numbers are referenced to Tables 7 and 13.

Peak 22 (t_R = 4.884 min, λ_{max} = 278 nm) had a $[M-H]^-$ at *m/z* 449. The MS/MS fragmentation pattern included ions at *m/z* 287 (dominant ion, M- 162 amu, loss of a hexose unit), *m/z* 193 and *m/z* 147 (Table 7). Though the dominant fragment ion matched the mass of eriodictyol, the UV-*vis* characteristics and fragmentation pattern of **peak 22** did not match a typical pattern for flavanones (Figure 9). **Peak 22** was thus denoted as unknown, but it could be related to eriodictyol. It was detected in TX430 (black) extract only.

Peak 24 (t_R = 9.365 min, λ_{max} = 287 nm) had a $[M-H]^-$ at *m/z* 287. The MS/MS fragmentation pattern included ions at *m/z* 151 and *m/z* 135 (Table 7), which matched the fragments of eriodictyol (*82*). In addition, the retention time of **peak 24** matched that of eriodictyol standard (9.395 min). Hence **peak 24** was identified as eriodictyol. It was only detected in SC748-H extract. Eriodictyol was the product of hydrolysis of eriodictyol glycosides in lemon-yellow sorghum extract SC748. It is a major peak in hydrolyzed lemon-yellow sorghum extract SC748-H due to the abundance of eriodictyol glycosides in SC748 (Figure 5).

Peak 25 (t_R = 10.227 min, λ_{max} = 279 nm) had a $[M-H]^-$ at *m/z* 287. The MS/MS fragmentation pattern included ions at *m/z* 151 and *m/z* 135 (Table 7). **Peak 25** had the mass of eriodictyol, but its UV-*vis* characteristics and fragmentation pattern did not match those of eriodictyol (Figure 9), thus **peak 25** was denoted as unknown, but could be related to eriodictyol. It was only detected in TX430 (black) extract.

Peak 30 (t_R = 12.422 min, λ_{max} = 289 nm) had a $[M-H]^-$ at *m/z* 271. The MS/MS fragments included a dominant ion at *m/z* 151 and a diagnostic ion at *m/z* 119 (M-152

amu) (Table 7), which matched the pattern for naringenin (*82*). In addition, the retention time of **peak 30** matched that of naringenin standard (12.452 min). Hence **peak 30** was identified as naringenin. It was detected in TX2911, 99LGWO50, and SC748-H extracts.

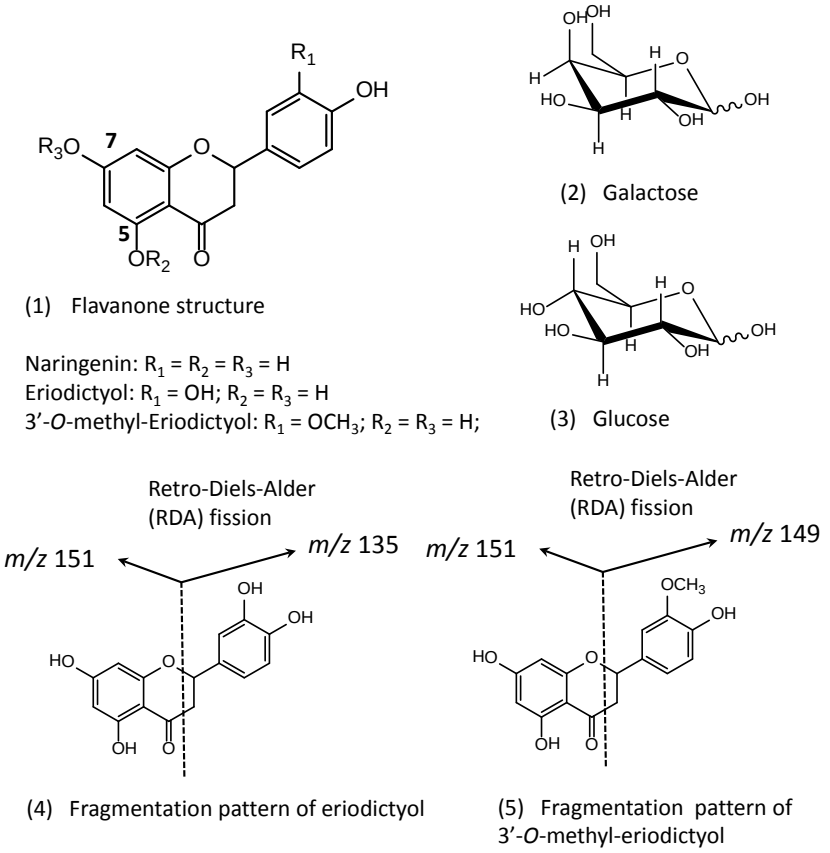


Figure 9. Chemical structures of flavanone monomers and their glycosyl derivatives detected in sorghum extracts.

Peak 31 (t_R = 13.073 min, λ_{max} = 326 nm) was a mixture and with a dominant $[M-H]^-$ at *m/z* 301. The MS/MS fragments pattern included ions at *m/z* 286 (M- 15 amu,

loss of a methyl unit) and *m/z* 151 (diagnostic ion of flavanones, fragment from Retro-Diels-Alder fission) (Table 7 and Figure 9). The mass and fragments matched the pattern for a –*O*-methyl- substituted derivative of eriodictyol. Based on the existence of ion at *m/z* 151, we inferred that the methyl group should be substituted in the B-ring (Figure 9). Hence **peak 31** was identified as 3'-*O*-methyl-eriodictyol or 4'-*O*-methyl-eriodictyol. It was only detected in SC748-H extract, most likely as a result of hydrolysis.

Peaks 26, 27, 28 (t_R = 10.962, 11.552, 11.813 min, λ_{max} = 295 nm) all had a $[M-H]^-$ at *m/z* 689. The MS/MS fragmentation pattern was almost identical among the three compounds, which included ions at *m/z* 401 (M- 288 amu), *m/z* 281, *m/z* 271, *m/z* 255, and *m/z* 125 (Table 7). Based on the UV-*vis* absorption pattern (Table 7), the structure should not have C2-C3 double bonds which would shift the λ_{max} to above 310 nm. These compounds are likely derivatives of pyrano-flavanones, based on similar pyrano-apigeninidin structure detected in red sorghum leaf sheath (*170*). The loss of 288 amu also matched the structures of common dimeric flavanone-flavanol molecules (*171*). Hence we inferred that these compounds are a catechin unit connected to a pyrano-naringenin unit by C3-C4 linkage (Figure 10) (*172*). Due to the existence of three chiral carbons, the three peaks may represent isomers of the pyrano-compound. Thus **peaks 26, 27, and 28** were tentatively identified as isomers of pyrano-naringenin-(3 → 4)-catechin. Other structure elucidation strategies, such as the NMR, are needed to confirm this structure identification. **Peaks 26 and 28** were only detected in dark red 99LGWO50 sorghum extract as decent peaks. **Peak 27** was detected as a major peak in dark red 99LGWO50 sorghum extract and a minor peak in bright red TX2911 sorghum extract.

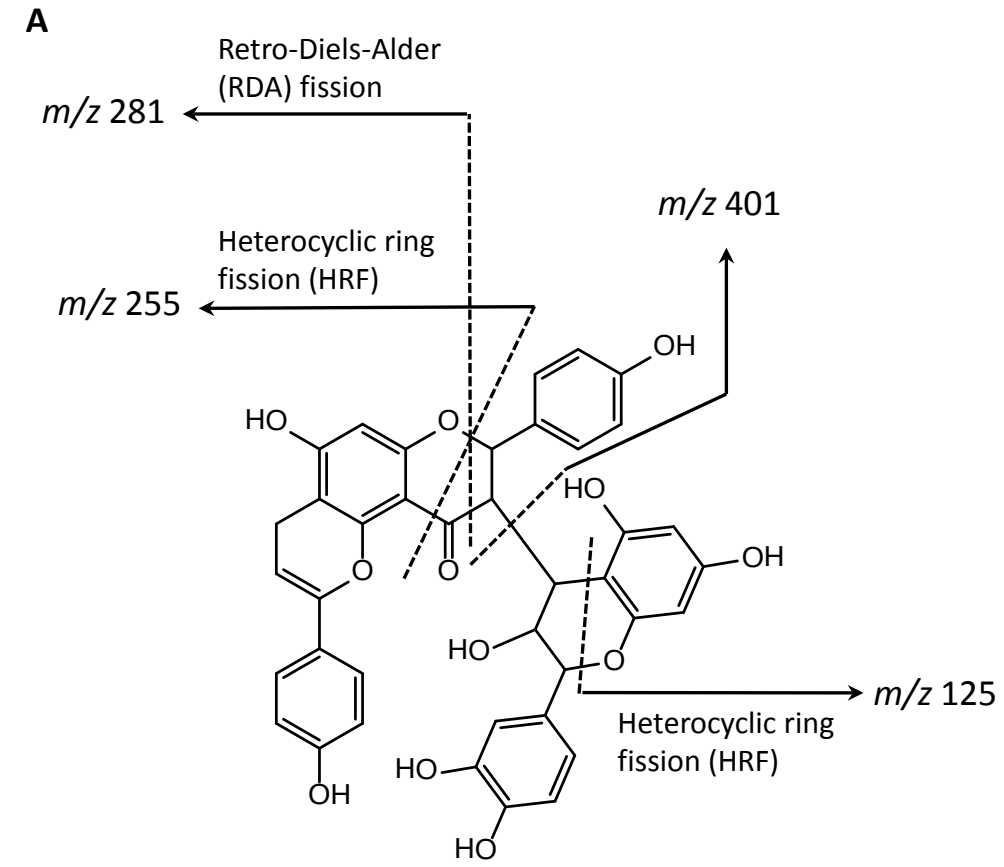
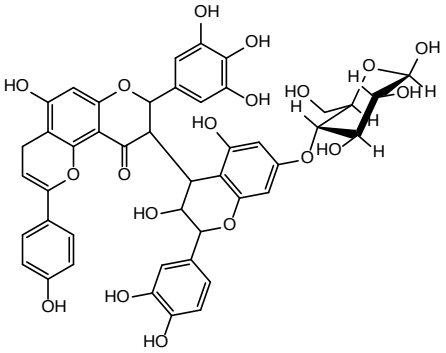


Figure 10. Schematic diagram showing fragmentation pattern of **peak 26**, **27**, or **28** (A); MS/MS fragmentation pattern of **peak 26** (B).

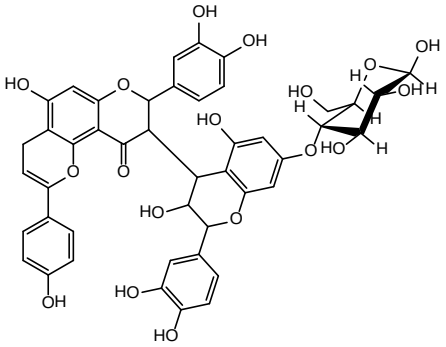
Peak 29 (t_R = 11.821 min, λ_{max} = 295 nm) had a $[M-H]^-$ at *m/z* 1107. The MS/MS fragments included major ions at *m/z* 705 (14% intensity, M-402 amu), *m/z* 401 (100% intensity, M-705 amu), *m/z* 417 (M- 402 amu – 288 amu) (Table 7), which indicated the inclusion of 417 amu unit, 401 amu unit, and a 288 amu unit. Based on previous elucidation pattern (Figure 10), **peak 29** was tentatively identified as a trimer of pyrano-naringenin-(3 → 7)-pyrano-eriodictyol-(3 → 4)-catechin (Figure 11). **Peak 29** was only detected in TX2911 extract.

Both **peak 32** (t_R = 13.843 min, λ_{max} = 302 nm) and **peak 33** (t_R = 14.097 min, λ_{max} = 302 nm) had $[M-H]^-$ at *m/z* 773. The MS/MS fragments included major ions at *m/z* 401 (100% intensity, M-372 amu), *m/z* 473, and *m/z* 209 (Table 7), which indicated the inclusion of 401 amu unit and 372 amu unit. Based on previous elucidation pattern (Figure 10), it is reasonable to believe that this 372 amu unit is a pyrano-flavonoid derivative. The λ_{max} (302 nm) of these two peaks were different from the other pyrano-flavanones (295 nm), which indicated that there may have a different pyrano conjugation pattern. The proposed structure was pyrano-3-deoxyflavan-3-ol; thus **peaks 32 and 33** were identified as pyrano-3-deoxyflavan-3-ol-(3 → 3)-pyrano-naringenin (Figure 11). The two peaks are probably isomers of this compound. Both **peaks 32 and 33** were only detected in 99LGWO50 extract.

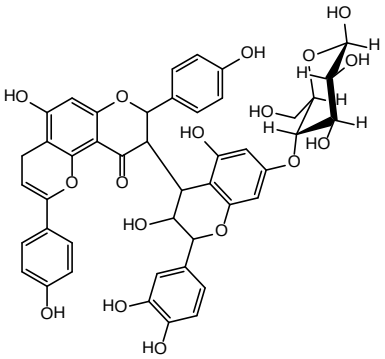
Glycosides of compounds proposed as pyrano-flavanone-flavanol dimers were also detected (**peaks 18, 21** and **23**; Figure 11).



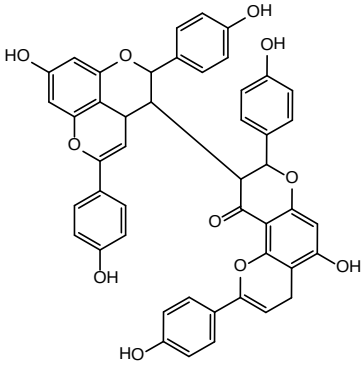
Peak 18 [M-H]$^-$ = 883
Pyrano-3', 4', 5', 5, 7-pentahydroxyflavanone-(3 → 4)-catechin-7-*O*-glucoside



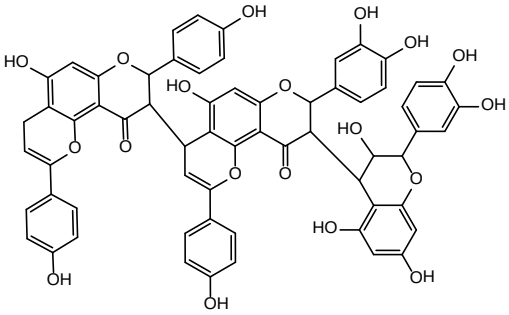
Peak 21 [M-H]$^-$ = 867
Pyrano-eriodictyol-(3 → 4)-catechin-7-*O*-glucoside



Peak 23 [M-H]$^-$ = 851
Pyrano-naringenin-(3 → 4)-catechin-7-*O*-glucoside



Peak 32 & 33 [M-H]$^-$ = 773
Pyrano-3-deoxyflavan-3-ol-(3 → 3)-pyrano-naringenin



Peak 29 [M-H]$^-$ = 1107
Pyrano-naringenin –(3 → 7)-pyrano-eriodictyol-(3 → 4)-catechin

Figure 11. Chemical structures of pyrano-flavanone derivatives detected in sorghum extracts.

Peak 18 (t_R = 3.535 min, λ_{max} = 284 nm) had a $[M-H]^-$ at *m/z* 883. The MS/MS fragments included major ions at *m/z* 721 (21% intensity, M- 162 amu, loss of a hexose unit), *m/z* 433 (100% intensity, M- 162 amu - 288 amu) as well as ions at *m/z* 287 (Table 7). The ion at *m/z* 433 represented an M+32 (2 –OH group) pattern compared with the dominant ion at *m/z* 401 of **peaks 26**, **27** and **28**. Thus **peak 18** was tentatively identified as pyrano-3′, 4′, 5′, 5, 7-pentahydroxyflavanone-(3 → 4)-catechin-7-*O*-glucoside dimer (Figure 11). **Peak 18** was only detected in TX430 (black) extract.

Peak 21 (t_R = 4.735 min, λ_{max} = 284 nm) had a $[M-H]^-$ at *m/z* 867. The MS/MS fragments included major ions at *m/z* 705 (19% intensity, M-162 amu, loss of a hexose unit), *m/z* 417 (100% intensity, M- 162 amu - 288 amu) as well as ions at *m/z* 271 (Table 7). The ion at *m/z* 417 represented an M+16 amu (-OH group) pattern compared with the dominant ion *m/z* 401 of **peaks 26**, **27** and **28**. Thus **peak 21** was tentatively identified as pyrano-eriodictyol-(3 → 4)-catechin-7-*O*-glucoside dimer (Figure 11). **Peak 21** was only detected in TX430 (black) extract.

Peak 23 (t_R = 6.552 min, λ_{max} = 283 nm) had a $[M-H]^-$ at *m/z* 851. The MS/MS fragments included major ions at *m/z* 689 (14% intensity, M- 162 amu, loss of a hexose unit) and *m/z* 401 (100% intensity, M- 162 amu - 288 amu) as well as ions at *m/z* 125 (Table 7). The ion at *m/z* 689 matched the fragmentation pattern of **peaks 26**, **27** and **28**; thus **peak 23** was tentatively identified as pyrano-naringenin-(3 → 4)-catechin-7-*O*-glucoside dimer (Figure 11). **Peak 23** was detected in TX2911 and TX430 (black) extracts. Further structure elucidation is needed for all the compounds that are proposed as pyrano-flavanone-flavanol derivatives.

Table 8. Contents (mg/g extract) of flavanone monomers in sorghum extracts as quantified by HPLC-DAD system[a].

Compound	Peak No.	TX2911 (red)	99LGWO50 (red)	SC748-H (hydrolyzed lemon-yellow)	SC748 (lemon-yellow)
Eriodictyol-5-*O*-galactoside[b]	16	ND	ND	0.69 ± 0.007	8.53 ± 0.36
Eriodictyol-7-*O*-galactoside[b]	17	ND	ND	1.21 ± 0.006	35.0 ± 1.1
Eriodictyol	24	ND	ND	11.0 ± 0.66	0.22 ± 0.005
Naringenin-5-*O*-galactoside[c]	19	ND	ND	0.94 ± 0.004	6.80 ± 0.18
Naringenin-7-*O*-galactoside[c]	20	0.52 ± 0.002	2.55 ± 0.29	1.83 ± 0.01	35.9 ± 1.00
Naringenin	30	ND	0.062 ± 0.014	12.2 ± 0.002	2.17 ± 0.06
3'-OMe-Eriodictyol[d]	31	ND	ND	0.24 ± 0.004	ND
Total flavanone monomers		0.52 ± 0.002	2.61 ± 0.30	28.1 ± 0.67	88.6 ± 2.70

[a]All values are expressed as mean ± SD of two separate runs. [b]Expressed as eriodictyol-7-*O*-glucoside. [c]Expressed as naringin (naringenin-7-*O*-rutinoside). [d]Expressed as eriodictyol. ND = not detected. Peak numbers are referenced to Figure 8.

Quantification of flavanones and their glycosyl derivatives in sorghum extracts. The contents of flavanone monomers and their glycosyl derivatives in sorghum extracts are shown in Table 8. Due to lack of standards and absolute certainly of the pyrano-flavanone structures, the oligomers were not quantified. Only TX2911, 99LGWO50, and SC748-H extracts contained detectable levels of flavanone monomers. Among the 3 sorghum extracts, the hydrolyzed lemon-yellow sorghum extract (SC748-H) had the highest amount of flavanone monomer derivatives (28.1 mg/g extract), consisting mainly of eriodictyol and naringenin aglycones. The Aglycones were products of acid hydrolysis (Figure 5). The red sorghum extracts, 99LGWO50 and TX2911 had 2.61 and 0.52 mg/g, respectively, of flavanone monomers, mostly as naringenin-7-*O*-galactoside.

The compositional differences among red and lemon-yellow sorghums were in accordance with Dykes et al. (*24, 25*).

Table 9. Identification of flavones in sorghum extracts (monitored at 340 nm) based on UPLC retention time (t_R), UV-*vis* spectroscopic characteristics (λ_{max}), and MS-MS/MS spectroscopic pattern. Ionization was performed in the negative mode. Peak numbers are referenced to Figure 6.

Peak No.	**t_R (min)**	**λ_{max} (nm)**	**$[M-H]^-$ (*m/z*)**	**MS/MS fragments (*m/z*)**	**Proposed Identification**
34	3.124	338	563	475, 395, 383, 353, 311, 264	6-C-glucosyl-8-C-arabinosyl-Apigenin
35	3.905	341	447	285	Luteolin-7-*O*-galactoside
36	5.073	346	447	285	Luteolin-7-*O*-glucoside
37	6.762	349	607	299, 284	Luteolin-3'-OMe-7-*O*-rutinoside
38	10.57	351	285	133	Luteolin
39	10.955	341	315	300, 228, 201, 137	3'-OMe- 4', 5', 5, 7-tetrahydroxylflavone
40	13.385	338	269	117, 135, 119	Apigenin
41	14.587	343	329	314, 299, 271, 243, 199, 133	Tricin (3', 5'-OMe-4', 5, 7-trihydroxylflavone)
42	18.5	340	343	313, 298, 270	3', 5'-OMe-4', 5, 6, 7-tetrahydroxyl flavone

Identification of flavones and their derivatives. Flavones identified in sorghum were mainly apigenin and luteolin derivatives (Table 9, Figure 6).

Peak 34 (t_R = 3.124 min, λ_{max} = 338 nm) had a $[M-H]^-$ at *m/z* 563. The MS/MS fragments included two major ions at *m/z* 383 (100% intensity) and *m/z* 353 (98% intensity) as well as ions at *m/z* 475, *m/z* 395, and *m/z* 311, which matched fragmentation pattern of *C*-glycosides (*95*) (Table 9). Based on mass and elution characteristics, **peak**

34 was identified as 6-*C*-glucosyl-8-*C*-arabinosyl apigenin (*95*) (Figure 12). **Peak 34** was only detected in White and 99LGWO50 extracts. The presence of 6-*C*-glucosyl-8-*C*-arabinosyl apigenin has been reported in millet grains (*95*), but not in sorghum.

Peak 35 (t_R = 3.905 min, λ_{max} = 341 nm) had a $[M-H]^-$ at *m/z* 447. **Peak 36** (t_R = 5.073 min, λ_{max} = 346 nm) had the same $[M-H]^-$ at *m/z* 447. The MS/MS of these two compounds included only a dominant ion at *m/z* 285 (M-162 amu, loss of a hexose unit), which matched fragmentation pattern of luteolin glycoside (Table 9). The retention time of luteolin-7-*O*-glucoside standard was 5.099 min, so **peak 36** was identified as luteolin-7-*O*-glucoside. Galactosides elute sooner than glucosides in reverse-phase HPLC (*166*), thus, **peak 35** was tentatively identified as luteolin-7-*O*-galactoside. Both luteolin glycosides were identified in SC748-H extracts, likely a consequence of incomplete hydrolysis of SC748.

Peak 37 (t_R = 6.762 min, λ_{max} = 349 nm) had a $[M-H]^-$ at *m/z* 607. The MS/MS fragmentation pattern included ions at *m/z* 299 (M-308 amu, loss of a rutinoside unit) and *m/z* 284 (M-308 amu -15 amu, loss of a –*O*-methyl unit of *m/z* 299) (Table 9). According to the mass and fragments, **peak 37** was identified as luteolin-3′-*O*-methyl-7-*O*-rutinoside. It was detected in TX430 (black) extract only.

Peak 38 (t_R = 10.570 min, λ_{max} = 351 nm) had a $[M-H]^-$ at *m/z* 285. The MS/MS fragments showed one dominant ion at *m/z* 133, which reflected the Retro-Diels-Alder fission of the C-ring. Base on authentic standard, **peak 38** was identified as luteolin (Table 9), and was detected in White, TX430 (black), 99LGWO50 and SC748-H extracts.

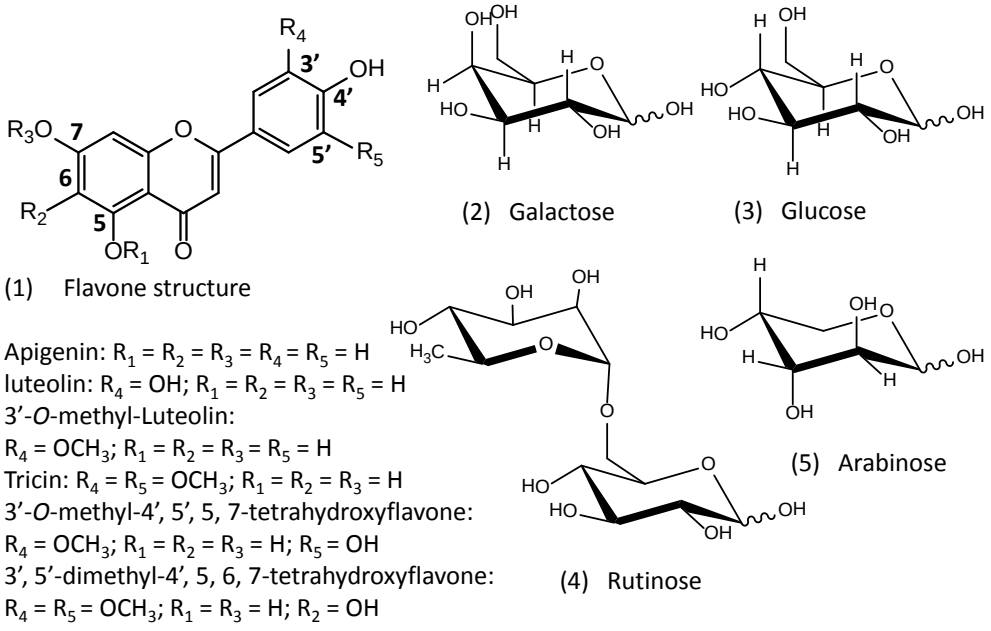


Figure 12. Chemical structures of flavones and their glycosyl derivatives identified in sorghum extracts.

Peak 40 (t_R = 13.385 min, λ_{max} = 338 nm) had a $[M-H]^-$ at *m/z* 269. The MS/MS fragments showed ions at *m/z* 117, *m/z* 135, and *m/z* 119, which reflected the Retro-Diels-Alder fission of the C-ring. Based on authentic standard, **peak 40** was identified as apigenin (Table 9), and was detected in White, TX430 (black), and 99LGWO50 extracts.

Besides flavones and their glycosides, various methoxylated flavones were also detected. **Peak 39** (t_R = 10.955 min, λ_{max} = 341 nm) had a $[M-H]^-$ at *m/z* 315. The MS/MS fragment included one dominant ion at *m/z* 300 (M-15 amu, loss of a methyl group) (Table 9). **Peak 41** (t_R = 14.587 min, λ_{max} = 343 nm) had a $[M-H]^-$ at *m/z* 329. The MS/MS fragments included ions at *m/z* 314 (M-15 amu, loss of a methyl group) and at *m/z* 299 (M-15 amu – 15 amu, loss of two methyl groups) (Table 9). **Peak 42** (t_R =

18.5 min, λ_{max} = 340 nm) had a $[M-H]^-$ at *m/z* 343. The MS/MS fragments included ions at *m/z* 313 (M-30 amu, loss of two methyl groups) and at *m/z* 298 (M-30 amu – 15 amu, loss of three methyl groups) (Table 9). The UV-*vis* spectroscopic characteristics indicated that these three compounds are flavone derivatives. Methoxylated flavone, tricin, was detected in millet grains (*95*), therefore, **peak 39** was identified as 3'-OMe- 4', 5', 5, 7-tetrahydroxyflavone (Figure 12), **peak 41** was identified as tricin (3', 5'-OMe-4', 5, 7-trihydroxyflavone) (Figure 12) and **peak 42** as 3', 5'-OMe-4', 5, 6, 7-tetrahydroxyl flavone (Figure 12). These three compounds were detected in TX430 (black) extract. Tricin (**peak 41**) was also detected in SC748-H extract.

Quantification of flavones in sorghum extracts. Flavones and their glycosyl derivatives were detected in all sorghum extracts except the red sorghum TX2911 extract. The 99LGWO50 extract had the highest amount of flavones (4.84 mg/g), followed by TX430 (black) (2.50 mg/g), White (2.27 mg/g), and the lemon-yellow extract SC748-H (1.12 mg/g). The White and 99LGWO50 extracts contained higher amount of apigenin and apigenin glycosides than luteolin. On the other hand, TX430 (black) and SC748-H extracts contained higher luteolin than apigenin derivatives. This difference was attributed to their secondary plant color. Sorghum varieties with tan secondary plant color accumulate more apigenin while those with purple secondary plant color accumulate more luteolin (*24, 25*). The methoxylated flavones were almost exclusively present in TX430 (black) sorghum extract, except that SC748-H extract contained a small amount of tricin.

Table 10. Contents (mg/g extract) of flavones and their glycosyl derivatives in sorghum extracts as quantified by HPLC-DAD system[a].

Compound	Peak No.	White	TX430 (black)	99LGWO50 (red)	SC748-H (hydrolyzed lemon-yellow)
Luteolin-7-*O*-galactoside[b]	35	ND	ND	ND	Trace
Luteolin-7-*O*-glucoside[b]	36	ND	ND	ND	0.592 ± 0.004
3'-OMe-Luteolin-7-*O*-rutinoside[b]	37	ND	0.246 ± 0.038	ND	ND
Luteolin	38	0.572 ± 0.176	0.528 ± 0.097	0.712 ± 0.089	0.444 ± 0.018
6-*C*-glucosyl-8-*C*-arabinosyl-Apigenin[c]	34	0.171 ± 0.053	ND	0.100 ± 0.015	ND
Apigenin	40	1.70 ± 0.59	0.223 ± 0.036	4.13 ± 0.17	Trace
3'-methoxy-4', 5', 5, 7-tetrahydroxyflavone[d]	39	ND	0.474 ± 0.087	ND	ND
Tricin[d]	41	ND	0.631 ± 0.168	ND	0.081 ± 0.003
3', 5'-methoxy-4', 5', 6, 7-tetrahydroxyflavone[d]	42	ND	0.395 ± 0.094	ND	ND
Total flavones		2.27 ± 0.77	2.50 ± 0.52	4.84 ± 0.26	1.12 ± 0.018

[a]All values are expressed as mean ± SD of two separate runs. [b]Expressed as luteolin-7-*O*-glucoside. [c]Expressed as apigenin-7-*O*-glucoside. [d]Expressed as luteolin. ND = not detected. Peak numbers are referenced to Figure 6.

Identification of 3-deoxyanthocyanidins. 3-Deoxyanthocyanidins were detected in the red and black pericap sorghum extracts. Five peaks of 3-deoxyanthocyanidins were identified as summarized in Table 11. **Peak 1a** (t_R = 3.311 min, λ_{max} = 487 nm, $[M+H]^+$ = 271) and **peak 2a** (t_R = 4.388 min, λ_{max} = 473 nm, $[M+H]^+$ = 255) was identified as luteolinidin and apigeninidin, respectively, by comparing with the retention times and mass with those of pure standards. **Peak 4a** (t_R = 5.584 min, λ_{max} = 460 nm) had a $[M+H]^+$ at *m/z* 309, which matched the mass of pyranoluteolinidin (Figure 13). This type of pigment is not naturally present in sorghum and is formed by reacting with acetone

(*173*), which was the extraction solvent. **Peak 5a** (t_R = 5.710 min, λ_{max} = 468 nm, $[M+H]^+$ = 269) showed MS/MS fragment at *m/z* 254 (M-15 amu, loss of a methyl group), which matched the fragmentation pattern of methoxylated apigeninidin. By confirming with the retention times of pure standards, peak 5a was identified as 7-*O*-methyl-apigeninidin. **Peak 3a** (t_R = 4.555 min, λ_{max} = 484 nm, $[M+H]^+$ = 285) had an MS/MS fragment at *m/z* 270 (M-15 amu, loss of a methyl group), which matched the fragmentation pattern of methoxylated luteolinidin. Thus **peak 3a** was identified as 7-*O*-methyl-luteolinidin based on evidence from previous study (*78*).

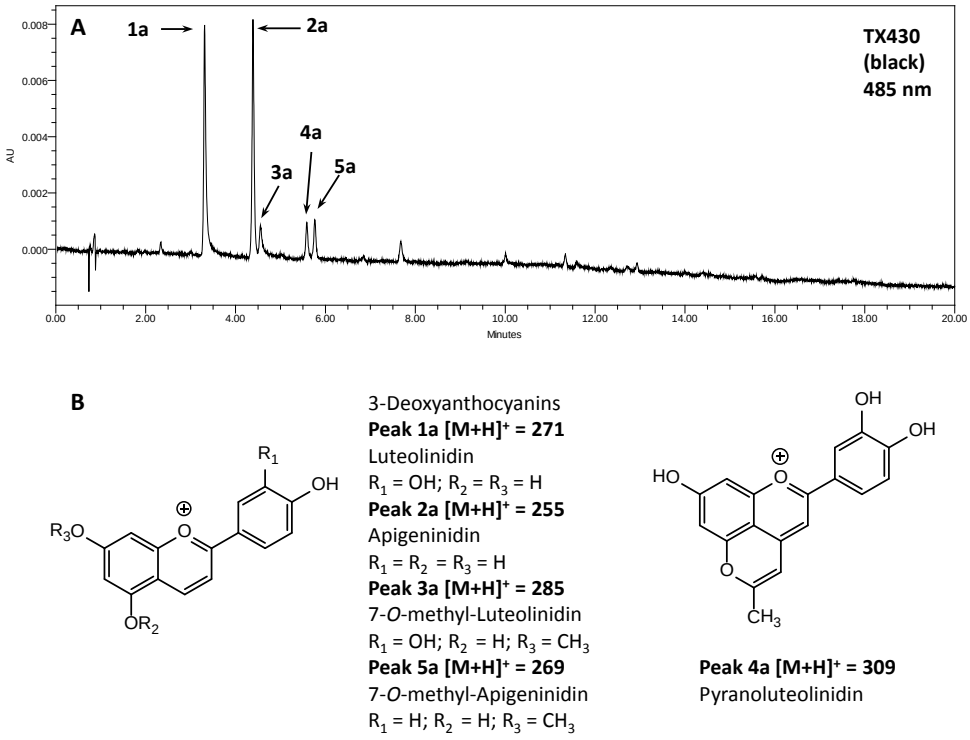


Figure 13. Reverse-phase UPLC chromatograms of 3-deoxyanthocyanins detected in TX430 (black) sorghum extracts at 485 nm (A) and chemical structures of these 3-deoxyanthocyanins (B). Peak numbers are referenced to Tables 11 and 12.

Table 11. Identification of 3-deoxyanthocyanidins in sorghum extracts (monitored at 485 nm) based on UPLC retention time (t_R), UV-*vis* spectroscopic characteristics (λ_{max}), and MS-MS/MS spectroscopic pattern. Ionization was performed in the positive mode. Peak numbers are referenced to Figure 13.

Peak No.	t_R (min)	λ max (nm)	$[M+H]^+$ (*m/z*)	MS/MS (*m/z*)	Proposed Identification
1a	3.311	487	271	n.f.	Luteolinidin
2a	4.388	473	255	n.f.	Apigeninidin
3a	4.555	484	285	270, 242	7-OMe-Luteolinidin
4a	5.584	460	309	n.f	Pyranoluteolinidin
5a	5.710	468	269	254, 226	7-OMe-Apigeninidin

n.f. = not fragmented.

Quantification of 3-deoxyanthocyanidins. The TX430 (black) sorghum extract had the highest amount of 3-deoxyanthocyanidins (3.04 mg/g), followed by the red TX2911 sorghum extract (1.29 mg/g) (Table 12). The 99LGWO50 red sorghum extract contained the least amount of 3-deoxyanthocyanidins (0.23 mg/g) (Table 12). The composition of 3-deoxyanthocyanidins was different among the 3 extracts: TX430 (black) had higher amount of luteolinidin than apigeninidin, while the 99LGWO50 extract contained exclusively apigeninidin and TX2911 extract contained similar amount of luteolinidin and apigeninidin. The difference in luteolinidin and apigeninidin composition can also be attributed to secondary plant color. Similarly to flavones, sorghum varieties with tan secondary plant color, e.g. 99LGWO50, accumulate more apigeninidin, and those with purple secondary plant color, e.g. TX430 (black), accumulate more luteolinidin (*25*).

Table 12. Contents (mg/g extract) of 3-deoxyanthocyanidins in sorghum extracts as quantified by HPLC-DAD system[a].

Compound	Peak No.	TX430 (black)	TX2911 (red)	99LGWO50 (red)
Luteolinidin (LUT)	1a	1.63 ± 0.17	0.303 ± 0.048	ND
Apigeninidin (APG)	2a	0.978 ± 0.12	0.496 ± 0.062	0.231 ± 0.076
7-OMe-LUT[b]	3a	0.200 ± 0.0018	0.222 ± 0.060	ND
7-OMe-APG	5a	0.231 ± 0.012	0.265 ± 0.023	ND
Total 3-deoxyanthocyanins		3.04 ± 0.29	1.29 ± 0.19	0.231 ± 0.076

[a]All values are expressed as mean ± SD from two separate runs. [b]Expressed as luteolinidin equivalent. ND = not detected. Peak numbers are referenced to Figure 13.

Identification of other phenolic compounds. Phenolic compounds other than phenolic acids, flavanones, or flavones, were also identified in sorghum grain extracts, but mostly as minor peaks (Table 13, Figures 6 & 8).

Peak 43 (t_R = 2.614 min, λ_{max} = 292/319 nm) had a $[M-H]^-$ at *m/z* 468. The MS/MS fragments included ions at *m/z* 306 (67% intensity, M-162 amu) and *m/z* 161 (35% intensity, M-307 amu). This fragmentation pattern matched that of N, N'-dicaffeoylspermidine (*174*). The dominant ion was at *m/z* 332 (M-136 amu), which represented the loss of a fragment of a caffeoyl unit without the carboxylic bonds (Table 13). Thus, we inferred that **peak 43** was N, N'-dicaffeoylspermidine, a polyamine (Figure 14). This compound was detected in all sorghum extracts. The presence of N, N′-dicaffeoylspermidine has been reported in eggplant pulps (*174*), but not in sorghum.

Peak 44 (t_R = 3.679 min, λ_{max} = 281 nm) had a $[M-H]^-$ at *m/z* 289. The MS/MS fragments included ions at *m/z* 151, *m/z* 137 (fragments of Retro-Diels-Alder fission), *m/z* 125 (dominant ion, fragment of heterocyclic fission), and *m/z* 109 (Table 13), which

matched the fragment pattern for catechin or epicatechin. Hence **peak 44** was proposed to be identified as (epi)catechin. **Peak 44** was only detected in TX430 (black) sorghum extract as a small peak.

Table 13. Identification of other phenolic compounds in sorghum extracts (monitored at 280 nm and 340 nm) based on UPLC retention time (t_R), UV-*vis* spectroscopic characteristics (λ_{max}), and MS-MS/MS spectroscopic pattern. Ionization was performed in the negative mode. Peak numbers are referenced to Figure 6 and Figure 8.

Peak No.	tR (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS/MS (*m/z*)	Proposed Identification
43	2.614	292/319	468	332, 306, 161, 135	N, N'-Dicaffeoylspermidine
44	3.679	281	289	151, 137, 125, 109	Catechin or epicatechin
45	5.169	295	177	177, 133, 107	Esculetin
46	5.991	287	303	285, 193, 177, 133, 125, 109	Taxifolin
47	6.848	379	449	287, 151	Eriodictyol-7-*O*-galactoside chalcone
48	7.378	377	301	191, 165, 161, 139, 109	7-OMe-Eriodictyol chalcone
49	8.205	289	287	195, 125	Unknown
50	8.221	371	433	271, 151	Naringenin-7-*O*-galactoside chalcone
51	9.521	370	285	191, 176, 165, 150	7-OMe-Naringenin chalcone

Peak 45 (t_R = 5.169 min, λ_{max} = 295 nm) had a $[M-H]^-$ at *m/z* 177. The MS/MS fragments included ions at *m/z* 133 and *m/z* 107 (Table 13), which matched the fragmentation pattern of esculetin (*175*). Therefore, **peak 45** was proposed to be esculetin (Figure 14). Esculetin is a lactone family compound commonly found in barks of herbs (*175*). Benincasa et al. (*176*) detected esculetin in the essential oils of lemon. **Peak 45** was detected only in SC748-H sorghum extract as a minor peak. Since the lemon-yellow SC748 sorghum extract (and the hydrolyzed extract SC748-H) contains high levels of flavanones, which are commonly found in citrus fruit, the detection of

esculetin in this sorghum extract suggests that esculetin and flavanones may share similar biosynthetic pathways.

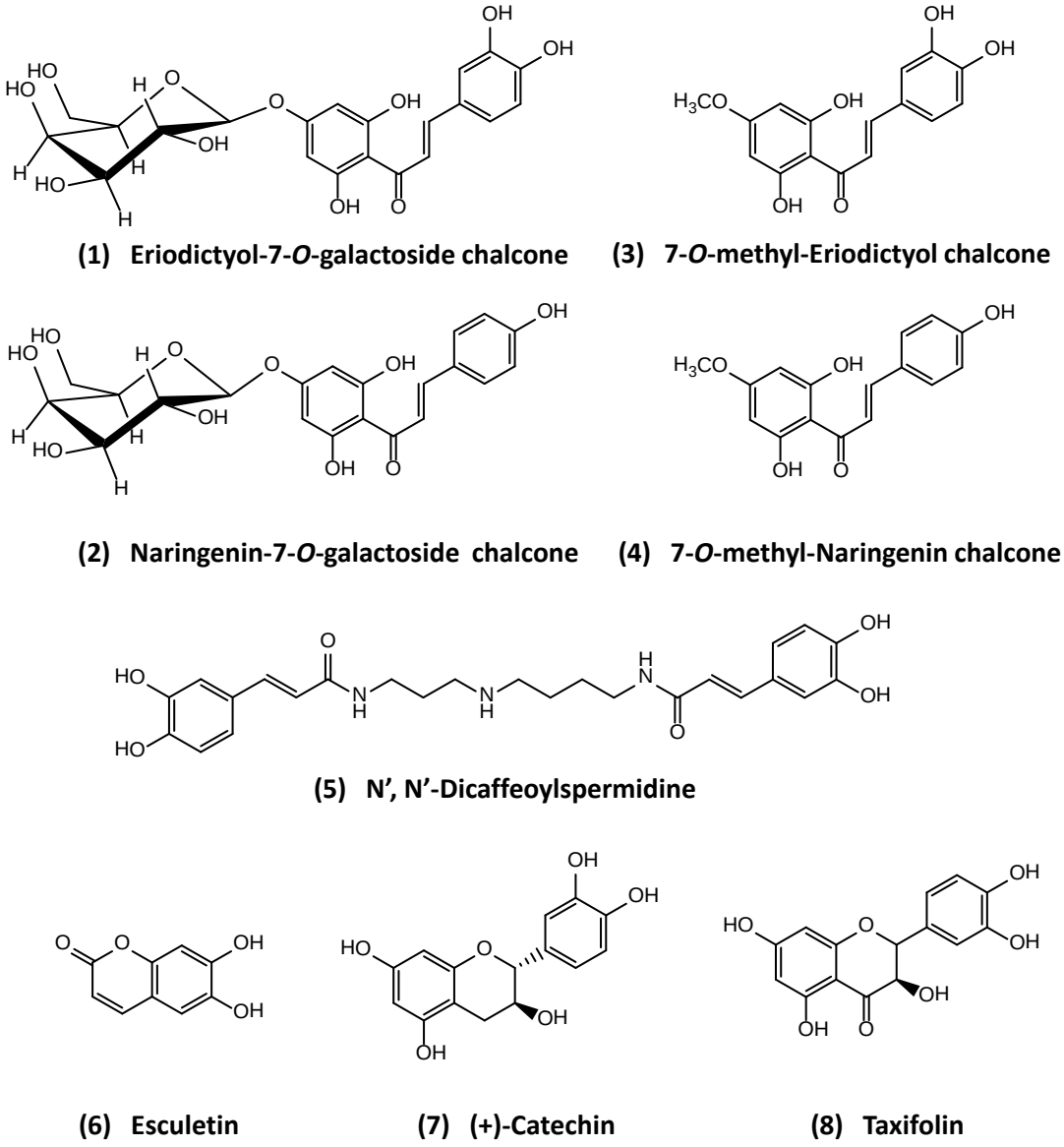


Figure 14. Chemical structures of minor phenolic compounds detected in sorghum extracts other than flavanones, flavones and phenolic acids.

Peak 46 (t_R = 5.991 min, λ_{max} = 287 nm) had a $[M-H]^-$ at *m/z* 303. The MS/MS fragments included ions at *m/z* 285 (5% intensity) and *m/z* 125 (100% intensity) (Table 13), which matched the fragment pattern of taxifolin (*95*). Hence **peak 46** was identified as taxifolin (Figure 14). Taxifolin belongs to flavanonol group. It was only detected in White sorghum extract as a small peak. The presence of taxifolin has been reported in millet grains (*95*).

Four peaks with λ_{max} ranging from 370-379 nm, which likely belong to chalcone derivatives, were identified. **Peak 47** (t_R = 6.848 min, λ_{max} = 379 nm) had a $[M-H]^-$ at *m/z* 449. **Peak 50** (t_R = 8.221 min, λ_{max} = 371 nm) had a $[M-H]^-$ at *m/z* 433. Both compounds had a dominant ion representing the loss of 162 amu (hexose unit): *m/z* 287 (eriodictyol unit) and *m/z* 271 (naringenin unit) (Table 13). In addition, **peak 47** and **peak 50** also had the same fragment ion at *m/z* 151. The UV-*vis* absorption pattern and the existence of fragment *m/z* 151 suggested they were chalcone glycosides. Chalcones are precursors of flavanones in flavonoids biosynthesis (*169, 177*). These two compounds were only detected in lemon-yellow sorghum extract (SC748 and its hydrolyzed SC748-H), which contained flavanone glycosides, eriodictyol and naringenin galactosides. Hence it is logical to identify **peak 47** as eriodictyol chalcone galactoside and **peak 50** as naringenin chalcone galactoside (Figure 14).

Peak 48 (t_R = 7.378 min, λ_{max} = 377 nm) had a $[M-H]^-$ at *m/z* 301. **Peak 51** (t_R = 9.521 min, λ_{max} = 370 nm) had a $[M-H]^-$ at *m/z* 285. Both peaks had fragment ions at *m/z* 165 (Table 13). Compared with the characteristic fragment of chalcone (*m/z* 151), ion *m/z* 165 had an increase of 14 amu, indicating existence of a methyl group. Based on

the fragment pattern, the methyl group should be on the A-ring; thus **peak 48** was identified as 7-*O*-methyl-eriodictyol chalcone and **peak 51** as 7-*O*-methyl-naringenin chalcone (Figure 14). Both **peak 48** and **peak 51** were only detected in TX430 (black) extract, most likely as intermediates of 3-deoxyanthocyanin biosynthesis (*169, 177*).

Table 14. Summary of compositions of phenolic compounds in sorghum extracts.

Compounds	White	TX2911 (red)	TX430 (black)	99LGWO50 (red)	SC748-H (hydrolyzed lemon-yellow)
Total phenolic acids[a]	26.8 ± 10.2	119.7 ± 10.8	4.25 ± 0.02	79.3 ± 10.9	5.10 ± 0.08
Total flavanone monomers[a]	N.D	0.52 ± 0.002	Trace	2.61 ± 0.30	28.1 ± 0.67
Total flavones[a]	2.27 ±0.77	N.D	2.50 ± 0.52	4.84 ± 0.26	1.12 ± 0.02
Total 3-deoxy-anthocyanins[a]	N.D	1.29 ± 0.29	3.07 ± 0.29	0.23 ± 0.08	N.D
Total phenols[b] (Folin-Ciocalteu method)	24.9 ± 4.3	88.4 ± 5.2	92.2 ± 4.3	103 ± 8.7	187.6 ± 2.6

[a]All values (mg/g) are expressed as mean ± SD of duplicate runs, referenced to Tables 6, 8, 10, 12. [b]Expressed as mg gallic acid equivalent per g of extract.

Relative composition of major groups of phenolics in the sorghum extracts. The contents of each major group of phenolic compounds in all 5 sorghum extracts are summarized in Table 14. The phenolic compositional differences were expected based on previous studies (*26*). All sorghum varieties contained phenolic acids (mainly as glycerol esters). The two red varieties TX2911 and 99LGWO had the highest concentrations of phenolic acids. The 3-deoxyanthocyanin pigments were only detected in the red and black sorghum varieties. TX430 (black) sorghum extract had the highest level of 3-deoxyanthocyanins. Flavanones (eriodictyol and naringenin derivatives) were detected as the major compounds in lemon-yellow sorghum extracts (SC748 and hydrolyzed

SC748). Naringenin glucosides were also detected in the red varieties TX2911 and 99LGWO50. White and TX430 (black) did not contain detectable flavanones. Flavones (luteolin and apigenin derivatives) were present in all sorghum extracts except the red TX2911 extract. The dark red sorghum extract 99LGWO50 contained the highest level of flavones. The phenolic profile information will be useful to explain differences in estrogenic activity.

Sorghum Extracts Promote the Growth of Human Breast Cancer Cells (MCF-7) Predominantly Expressing ERα

The MCF-7 cell line was derived from estrogen dependent human breast adenocarcinoma, and ERα is the predominant ER in these cells. It is widely used to screen and identify estrogenic compounds *in vitro*. Cells were cultured in charcoal-dextran treated FBS media for 48 hr before administration of treatment to deplete estrogen naturally present in the media, hence the estrogen added into the media was the only source of growth stimuli for MCF-7 cells. Estrogenic activity was shown as promotion of MCF-7 growth, similar to a physiological dose of estradiol (1 nM).

Three sorghum extracts, White, TX2911, and TX430 (black), at concentrations of 1-150 µg/mL were tested for their estrogenic potential in MCF-7 cells. The White and TX430 (black) sorghum extracts promoted the growth of MCF-7 cells in a dose-response manner; while red TX2911 sorghum extract did not (Figure 15). The TX430 (black) sorghum extract significantly promoted MCF-7 cell growth, as 1 nM estradiol, at 5 µg/mL. At the highest concentration tested (150 µg/mL), the TX430 (black) sorghum

extract showed a 2.3-fold increase in MCF-7 growth. The White sorghum extract showed less potency in promoting MCF-7 growth: The lowest concentration observed for a significant increase in cell growth was 50 µg/mL (38.6% increase). In general, the red TX2911 sorghum extract increased MCF-7 growth by 29.4-38.4%, which was only significant ($p = 0.048$) at the highest concentration (150 µg/mL). Thus in comparison to the effect of 1 nM estradiol (54.5%), it was concluded that TX2911 extract did not promote MCF-7 growth in a practically meaningful way.

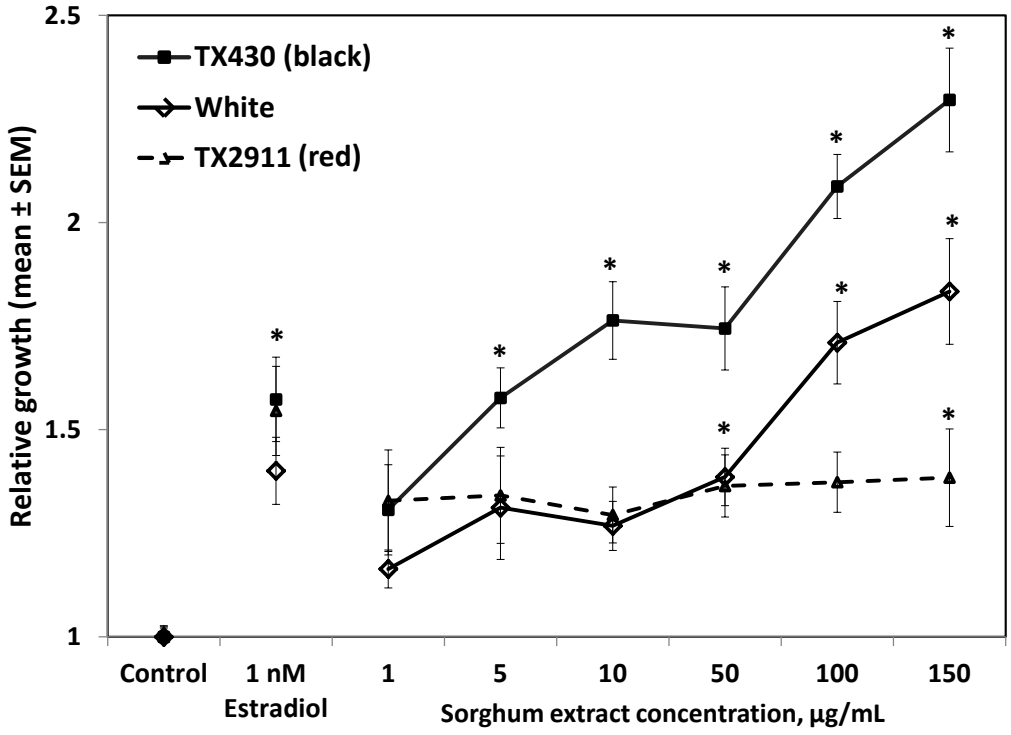


Figure 15. The effect of sorghum extracts, White, TX2911, and TX430 (black) on the growth of MCF-7 cells. Data are expressed as mean ± SEM from three separate experiments. Dunnett's t test was used to compare the least square means of each treatment with control post a one-way ANOVA analysis. Treatments with an asterisk indicate significant difference from the control (DMSO) ($p < 0.05$).

All sorghum extracts contained phenolic acids and their glycerol esters (Table 6). However, generally phenolic acids are not recognized as estrogenic, so the differences in phenolic acid profiles most likely did not contribute to the differences in estrogenic potential. The White and TX430 (black) sorghum extracts which promoted MCF-7 growth, contained similar concentrations of flavones (primarily luteolin and apigenin derivatives): TX430 (black) had 2.50 mg/g and White extract had 2.27 mg/g (Table 10). The red TX2911 sorghum extract, on the other hand, did not contain any flavones. However, it contained the flavanone, naringenin-7-*O*-galactoside (0.52 mg/g, Table 8). Naringenin was reported as an ERα antagonist, and is believed to antagonize the estrogen action in ERα-dependent cancers (*135*). Apigenin and luteolin, in contrast, were reported as agonists of ERα (*178*). This may explain why TX2911 extract did not promote growth of MCF-7 cells, whereas TX430 (black) and White extracts did. Another difference in phenolic composition among the 3 extracts was the content of 3-deoxyanthocyanins. The TX430 (black) extract had the highest 3-deoxyanthocyanins (3.04 mg/g, Table 12), followed by the TX2911 sorghum extract (1.29 mg/g, Table 12), and the White sorghum extract did not have any pigments. Whether 3-deoxyanthocyanins contributed to MCF-7 growth promoting effect was not clear. However, considering that TX430 (black) extract exerted a stronger potency than the White extract, it is possible that the 3-deoxyanthocyanin pigments could possess estrogenic activity.

Sorghum Extracts Induce the Transcriptional Activity of ERE-luciferase Reporter

The MCF-7 cells were transfected with ERE-luciferase reporter gene to monitor the transcriptional activity mediated through ERα. Administration of estradiol increases the activity of luciferase, which reflects the genomic action of estrogen via activation of ER and corresponding ERE binding. The White and TX430 (black) sorghum extracts (5, 10, 100 μg/mL) were selected to test with ERE-luciferase reporter to confirm the growth promoting effect in MCF-7 cells was mediated through ER. The TX430 (black) extract induced luciferase activity by 57% at 5 μg/mL, and 67% at 10 μg/mL; White sorghum extract induced 66% luciferase activity at 10 μg/mL, which were similar to the effect of 1 nM estradiol (67%). Other concentrations tested did not show any significant increase in luciferase activity (Figure 16). The results suggested that the growth promoting effect of White and TX430 (black) sorghum extracts observed in MCF-7 cells was possibly mediated through ERα.

The TX430 (black) sorghum extract induced luciferase activity at 5 μg/mL while the White sorghum extract at the same concentration did not. This evidence again suggested some groups of compounds in the TX430 (black) extract exerted higher estrogenic potency. Besides the 3-deoxyanthocyanins, TX430 (black) extract also had higher levels of methoxylated flavones and luteolin, but had lower level of apigenin than White sorghum extract (Table 10). Hydroxylation and methoxylation could enhance apoptosis inducing (*179*) and antiproliferative potential (*180*) of flavones. It is possible that these methoxylated flavones in TX430 (black) sorghum extract contributed to higher estrogenic activity, but additional investigations are needed.

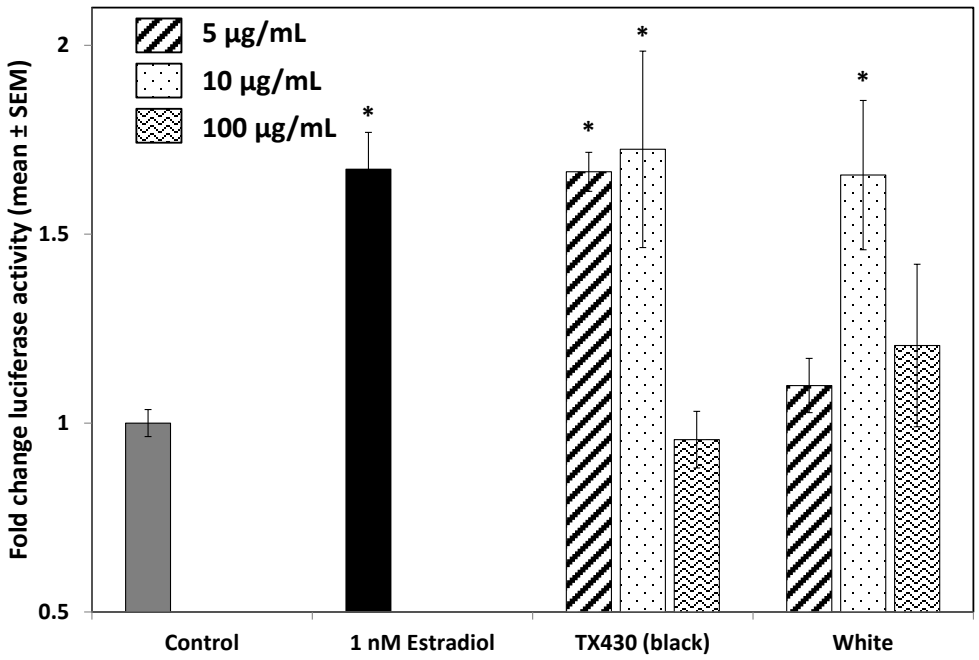


Figure 16. The effect of sorghum extracts, TX430 (black) and White (5, 10, 100 µg/mL), on transcriptional activity of ERE-luciferase reporter transfected in MCF-7 cells. Data are expressed as mean ± SEM from two separate experiments. Dunnett's t test was used to compare the least square means of each treatment with control post a one-way ANOVA analysis. Treatments with an asterisk indicate significant difference from the control (DMSO) ($p < 0.05$).

Sorghum Extracts Inhibit the Growth of Non-Malignant Young Adult Mouse Colonocytes (YAMCs) Predominantly Expressing ERβ

The non-malignant young adult mouse colonocytes (YAMCs), when cultured at non-permissive conditions, is a suitable *in vitro* model to represent the physiology (life cycle) of normal colonic epithelium. It undergoes slow proliferation for 24 hr and cell death in the following 5-8 days (*141*); hence has been used as a model to study physiological changes during initiation and promotion stages of colon carcinogenesis. The YAMC cells express predominantly ERβ. Estradiol, administered at non-permissive

conditions, inhibits the growth of YAMC cells (*11, 12*). The induction of apoptosis and activation of tumor suppressor gene *p53*, are proposed as mechanisms to protect the damaged colonocytes from proliferating into malignancy (*11, 12*).

In order to evaluate if sorghum extracts exert estrogenic activity and associated beneficial mechanisms through activation of ERβ, the effect of White, TX2911, and TX430 (black) sorghum extracts on growth of YAMC cells was determined. All three sorghum extracts dose-dependently inhibited YAMC cell growth at concentrations of 1-100 μg/mL, which suggested potential estrogenic activity (Figure 17). The TX2911 extract showed significant reduction of YAMC cell growth (20%) at 1 μg/mL, the lowest concentration tested, which was similar to the effect of 1 nM estradiol (17% reduction); at this concentration White and TX430 (black) extracts decreased YAMC cell growth by 8% and 11%, respectively. The White and TX430 (black) sorghum extracts significantly reduced YAMC growth by 16% and 19%, respectively, at the concentration of 5 μg/mL.

In order to confirm that the growth reduction effect of sorghum extracts was mediated through ER, ER antagonist ICI 182, 780 (ICI, 1 μM) was used to treat YAMC cells with these sorghum extracts simultaneously. ICI binds to ER competitively then blocks the binding of estrogenic compounds to ER. The concentrations chosen for this experiment were 5 μg/mL for TX430 (black) and TX2911 extracts, and 10 μg/mL for White sorghum extract, for their consistent similar growth inhibitory effect as estradiol. Co-treatment of ICI with estradiol did not affect the growth of YAMC cells (Figure 18). Similarly, co-treatment of ICI reversed the growth reduction effect of White and TX430 (black) sorghum extracts, which suggested the effect was mediated through ERβ (Figure

18). Interestingly, ICI co-treatment did not completely reverse the growth reducing effect of TX430 (black) sorghum extract ($p < 0.22$, Figure 18), which suggested some of the compounds in TX430 (black) extract inhibited YAMC growth through a mechanism other than the activities mediated through ER. On the other hand, the growth reducing effect of TX2911 extract on YAMC cell growth was not reversed by co-treating ICI, which indicates its antiproliferative effect was not mediated by ER.

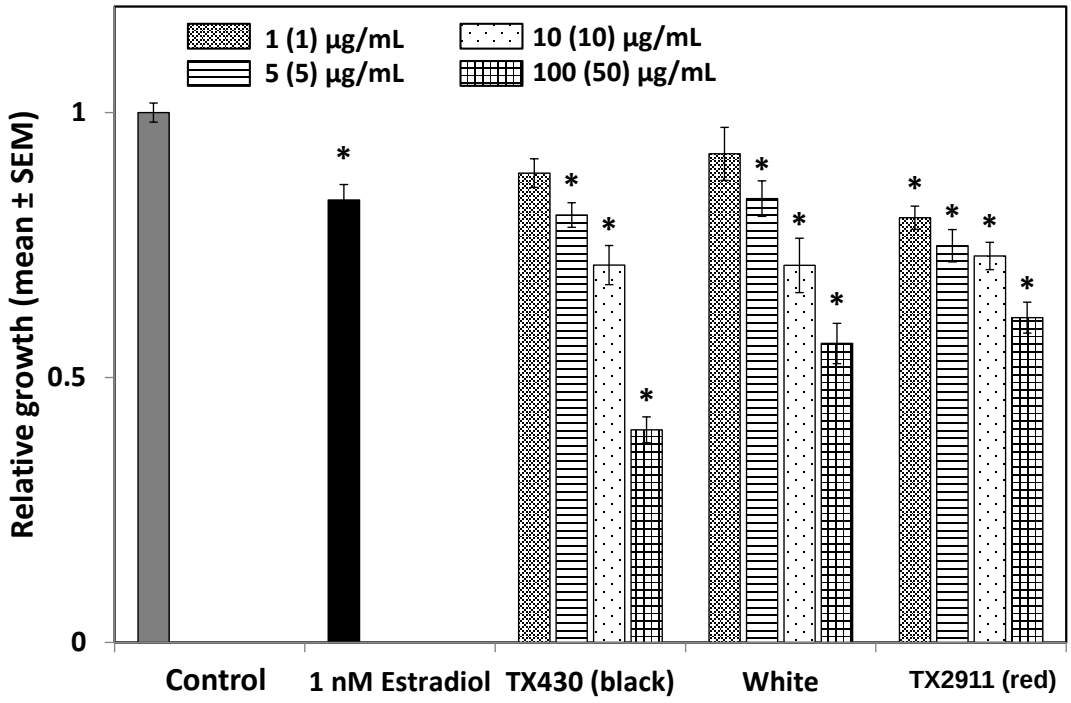


Figure 17. The effect of sorghum extracts, White, TX2911, and TX430 (black) at 1, 5, 10, 50 or 100 µg/mL, on the growth of non-malignant young adult mouse colonocytes (YAMCs). Data are expressed as mean ± SEM from three separate experiments. Dunnett's t test was used to compare the least square means of each treatment with control post a one-way ANOVA analysis. The concentrations of TX2911 extracts are shown in parentheses. Treatments with an asterisk indicate significant difference from the control (DMSO) ($p < 0.05$).

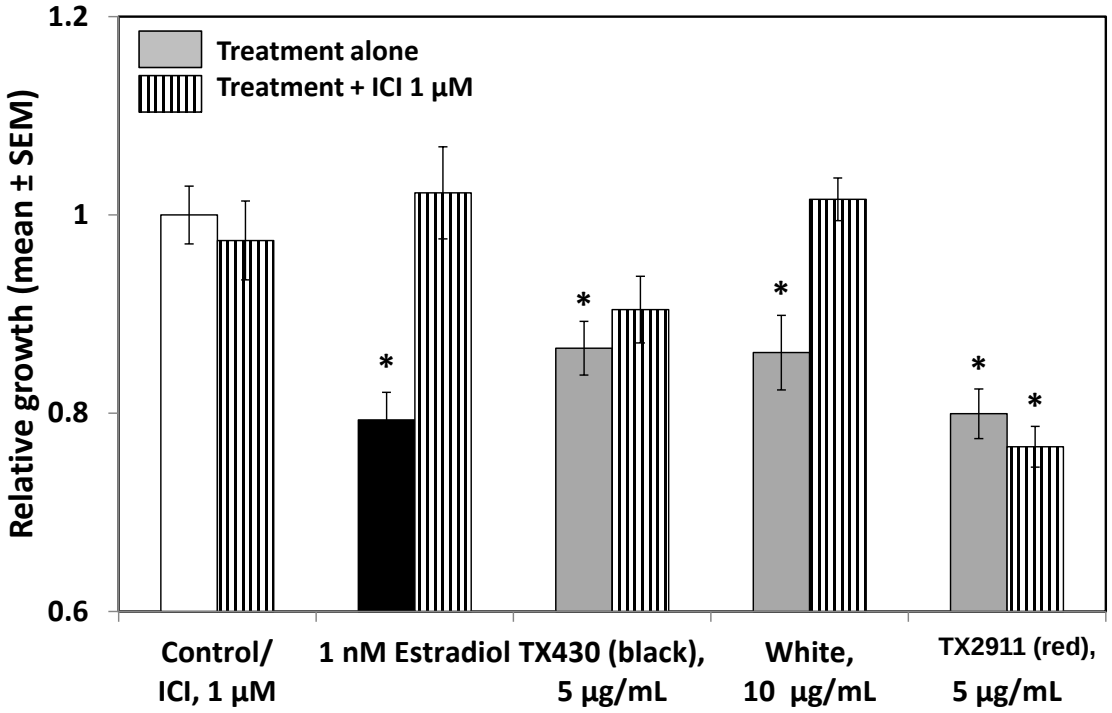


Figure 18. The effect of co-treatment of 1 µM ICI 182, 780 (estrogen receptor antagonist) with sorghum extracts, White (10 µg/mL), TX2911 (5 µg/mL), and TX430 (black) (5 µg/mL), on the growth of non-malignant young adult mouse colonocytes (YAMCs). Data are expressed as mean ± SEM from three separate experiments. Dunnett's t test was used to compare the least square means of each treatment with control post a one-way ANOVA analysis. Treatments with an asterisk indicate significant difference from the control (DMSO) ($p < 0.05$).

The red TX2911 sorghum extract did not show estrogenic potential in cell models that predominantly express either ERα or ERβ. Compared with the phenolic compositions of White and TX430 (black) sorghum extracts, TX2911 extract does not contain any detectable flavones, but contains naringenin-7-*O*-galactoside (Table 8 and Table 10). Flavones (apigenin and luteolin) (*178*), as well as naringenin (*135*) have been reported as ERα agonist and antagonist, respectively. Apigenin possesses stronger

estrogenic potency on ERα (tested with MCF-7 cells) than luteolin: at 10 μM, apigenin presents 60% of transcriptional activity shown by 1 nM estradiol while luteolin presents 20% (*178*). On the other hand, naringenin did not increase MCF-7 cell proliferation up to 1 μM but was able to interfere with the proliferation promoting effect of 1 nM estradiol at 1 μM (*135*). In a different study (conducted by Harris et al.), both apigenin and naringenin induced higher ERβ transcriptional activity than activity induced by binding to ERα in human breast cancer cells (MCF-7) co-transfected with ERα and ERβ (*31*). However apigenin exerted higher estrogenic potency than naringenin. Compared to 1 μM estradiol, apigenin (10 μM) showed 110% of the transcriptional activity via ERβ and about 70% via ERα, while naringenin (10 μM) showed approximately 70% and 30%, respectively (*31*). Thus it is logical to infer that the varietal differences in estrogenic activity among the White, red TX2911 and TX430 (black) sorghum extracts may be attributed to the compositional differences of flavones and flavanones. However, further investigation is needed to confirm the effect of flavones and flavanones on estrogenic activity of sorghum.

Besides flavones, the 3-deoxyanthocyanins are possible agonists of both ERα and ERβ. Considering the TX430 (black) extract generally showed estrogenic activity at lower concentrations than White sorghum extract, the 3-deoxyanthocyanin pigments may have contributed to estrogenic activity of TX430 (black) extract. However, the red TX2911 sorghum extract which also contained 3-deoxyanthocyanins did not show estrogenic potential. Thus the roles of the sorghum 3-deoxyanthocyanins and flavanones in estrogenic activity deserve further investigation.

Sorghum Extracts Induce Apoptosis Mediated through ER in Non-Malignant Young Adult Mouse Colonocytes (YAMCs)

Estradiol induces apoptosis in YAMC cells after activation of ERβ, which results in corresponding suppression of cell growth. This was proposed as a mechanism for chemoprevention of estradiol because it protects damaged colonocytes from growing into malignancy (*11*).

In order to further determine the mechanism of growth inhibition shown in YAMC cells, the capacity of White (10 μg/mL) and TX430 (black) (5 μg/mL) sorghum extracts to induce apoptosis (by measuring activity of caspase-3) was tested in YAMC cells with and without co-treatment of ER antagonist ICI. At the concentrations tested, White and TX430 (black) sorghum extracts increased the activity of caspase-3 by 38.9% and 29.7%, both comparable to 1 nM estradiol (32.0%) (Figure 19). The results showed that sorghum extracts induced apoptosis in YAMC cells. Co-administration of 1 μM ICI reversed the activity of caspase-3 to the levels similar to control. The ICI did not block the effect of White sorghum extract completely, which may indicate that some of the apoptotic activity induced by White sorghum extract was not mediated through ER.

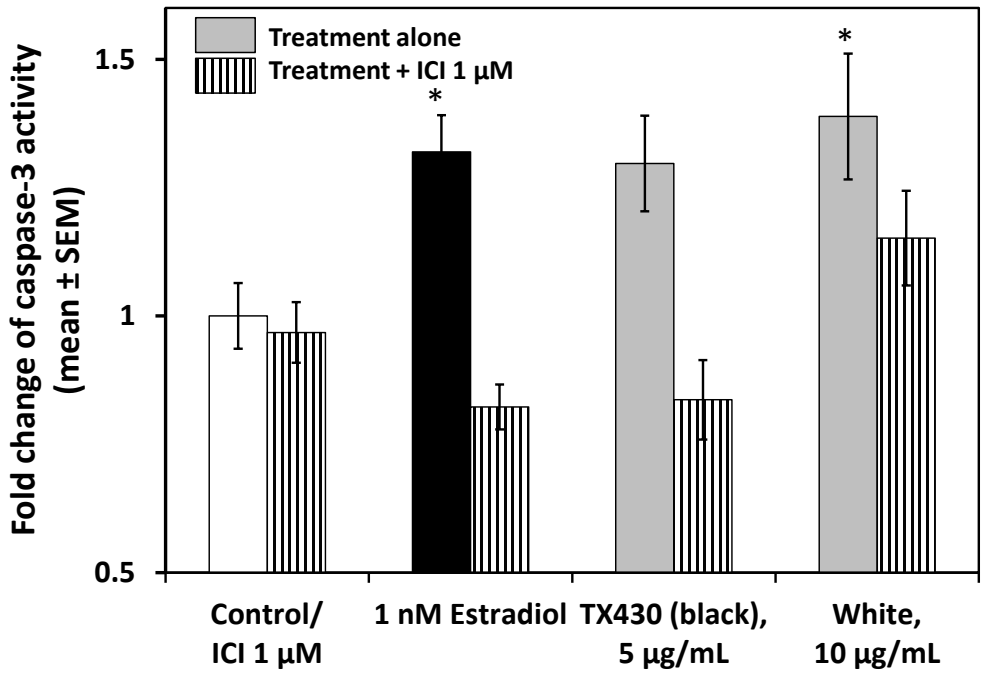


Figure 19. The effect of White (10 µg/mL) and TX430 (black) (5 µg/mL) sorghum extracts, alone or co-treated with 1 µM ICI 182, 780 (estrogen receptor antagonist), on the activity of caspase-3 in non-malignant young adult mouse colonocytes (YAMCs). Data are expressed as mean ± SEM from three separate experiments. Dunnett's t test was used to compare the least square means of each treatment with control post a one-way ANOVA analysis. Treatments with an asterisk indicate significant difference from the control (DMSO) ($p < 0.1$). The *p*-value of treatment alone is 0.055, 0.11, and 0.01 for estradiol, TX430 (black), and White sorghum extract, respectively; all other *p*-values are greater than 0.58.

Estrogenic Properties of 3-Deoxyanthocyanidins

To determine the estrogenic potential of sorghum 3-deoxyanthocyanins, pure apigeninidin and its methoxylated derivatives were tested for their growth reduction effect in YAMC cells (predominantly expressing ERβ), at concentrations of 0.01-100 µM. Growth reduction of YAMC cells was only observed at relatively high concentrations, i.e. 50 and 100 µM for apigeninidin and 7-*O*-methyl-apigeninidin, 5 µM

for 5,7-*O*-methyl-apigeninidin (Figure 20). Growth inhibition at such high concentrations is probably due to cytotoxic effects (*78*). Hence the 3-deoxyanthocyanins most likely were not estrogenic and did not contribute to the estrogenic activity of TX430 (black) sorghum extract. This also agrees with the fact that the red sorghum TX2911 which also contained 3-deoxyanthocyanins did not show estrogenic activity. Hence we may conclude that the most likely ER agonists in the sorghum varieties tested were the flavones. However, impact of flavanones on estrogenic activity of sorghum needs further investigation.

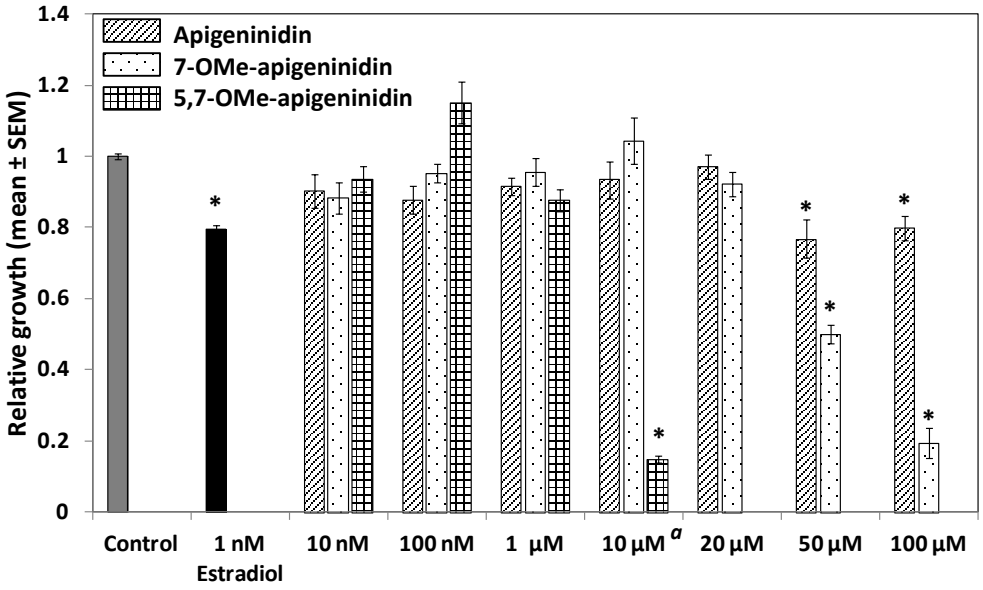


Figure 20. The effect of apigeninidin, 7-*O*-methyl-apigeninidin, and 5,7-*O*-methyl-apigeninidin (0.01-100 µM) on the growth of non-malignant young adult mouse colonocytes (YAMCs). [a]The concentration of 5,7-*O*-methyl-apigeninidin is 5 µM. Data are expressed as mean ± SEM from three separate experiments. Dunnett's t test was used to compare the least square means of each treatment with control post a one-way ANOVA analysis. Treatments with an asterisk indicate significant difference from the control (DMSO) ($p < 0.05$).

Sorghum Extracts High in Flavones or Flavanones Inhibit the Growth of Non-Malignant Young Adult Mouse Colonocytes (YAMCs) Predominantly Expressing ERβ

The potential contribution of flavones and flavanones to estrogenic activity of sorghum extracts was further investigated by two additional sorghum varieties with contrasting flavone and flavanone compositions. The dark red 99LGWO50 sorghum extract contained both flavones (4.84 mg/g total), especially apigenin (4.13 mg/g, Table 10), and flavanones (naringenin and its glycosides, 2.61 mg/g total, Table 8). On the other hand, the lemon-yellow sorghum extract (SC748) contained very high amount of flavanone glycosides and relatively low levels of flavones (Figure 5). In order to better evaluate the potential ERβ activation effect of flavanone aglycones, the lemon-yellow sorghum extract was hydrolyzed to obtain flavanone aglycones. The hydrolyzed lemon-yellow sorghum extract (SC748-H) contained very high amount (28.1 mg/g total) of flavanones (naringenin and eriodictyol, Table 8) but relatively low levels (1.12 mg/g total) of flavones (mainly luteolin and its glycosides, Table 10). The compositional differences allowed investigation of the estrogenic potential of flavones in the presence of flavanones, which have been suggested as ER antagonists in some studies.

The 99LGWO50 extract inhibited the growth of YAMC cells in a dose-dependent manner (Figure 21). It significantly reduced YAMC cell growth by 20.5% at 5 μg/mL; at 10 μg/mL, the inhibition was 25.3%. Similar trend was observed for the hydrolyzed lemon-yellow sorghum extract (SC748-H). However, the non-hydrolyzed lemon-yellow sorghum extract (SC748) did not show significant growth inhibitory effect in YAMC cells at 5 μg/mL and showed inhibitory effect similar to 1 nM estradiol only at

the highest concentration tested (50 µg/mL). These results suggest that both flavones and flavanones are estrogenic and that flavanone glycosides (predominant in the SC748 extract) have a lower potency.

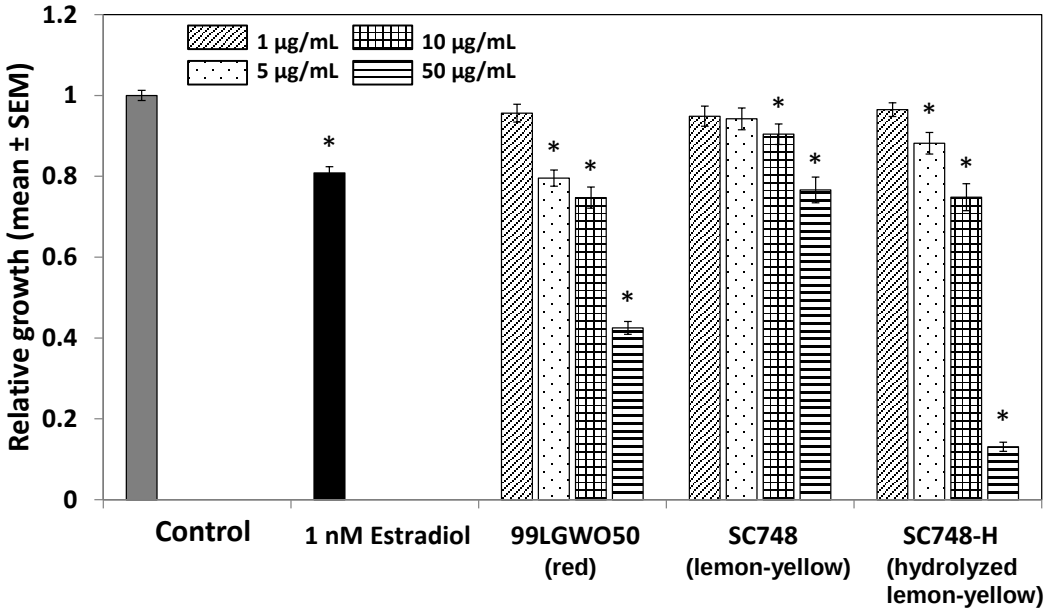


Figure 21. The effect of red sorghum 99LGWO50 extract (high in flavones), lemon-yellow sorghum extract SC748 (high in flavanone glycosides), and SC748-H (high in flavanone aglycones) (1-50 µg/mL) on the growth of non-malignant young adult mouse colonocytes (YAMCs). Data expressed as mean ± SEM from three separate experiments. Dunnett's t test was used to compare the least square means of each treatment with control post a one-way ANOVA analysis. Treatments with an asterisk indicate significant difference from the control (DMSO) ($p < 0.05$).

Considering the effect produced by 1 nM estradiol (19.1%), 5 µg/mL of red sorghum 99LGWO50 and hydrolyzed lemon-yellow sorghum (SC748-H) extracts were selected to confirm whether the growth inhibition was mediated through ER. Co-

treatment of ICI with both 99LGWO50 and SC748-H extracts reversed the inhibitory effect on YAMC cell growth, which suggests that ERβ mediated this physiological change (Figure 22). Harris et al. (*31*) showed that both flavones and flavanones can bind to ERβ and exert transcriptional activities in MCF-7 cells co-transfected with ERα and ERβ. Our findings indicate that both flavones and flavanones are likely agonists of ERβ in YAMC cells.

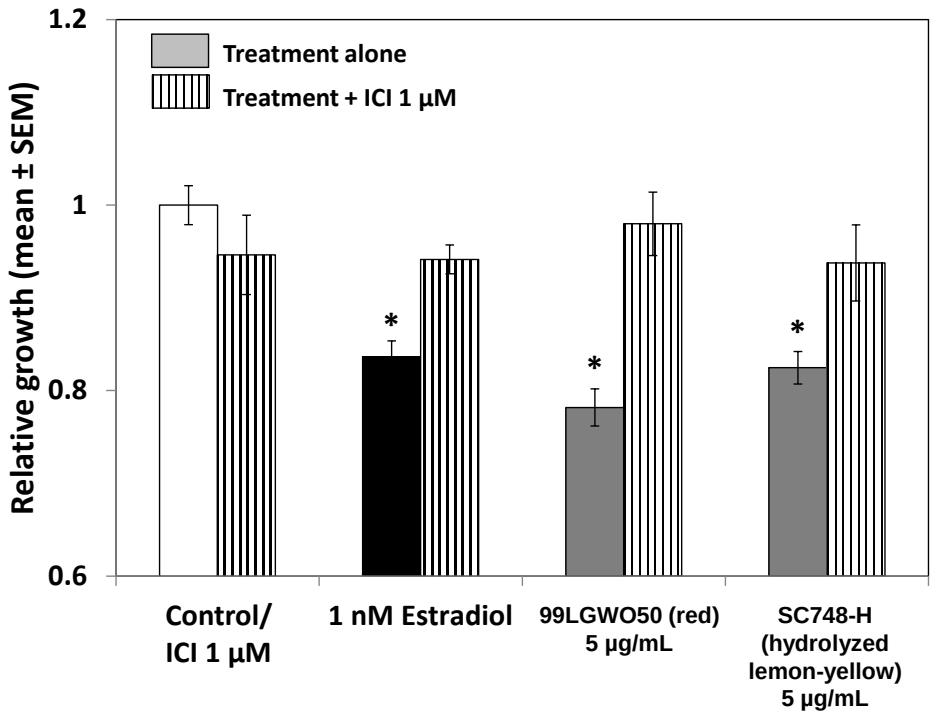


Figure 22. The effect of co-treatment of 1 µM ICI 182, 780 (estrogen receptor antagonist) with 5 µg/mL of sorghum extracts, 99LGWO50 (red with flavones) and SC748-H (lemon-yellow with flavanone aglycones), on the growth of non-malignant young adult mouse colonocytes (YAMCs). Data are expressed as mean ± SEM from three separate experiments. Dunnett's t test was used to compare the least square means of each treatment with control post a one-way ANOVA analysis. Treatments with an asterisk indicate significant difference from the control (DMSO) ($p < 0.05$).

It is interesting to note that the growth inhibition and estrogenic potency of SC748-H extract was consistently lower than that of 99LGWO50 extract (Figure 21 and Figure 22). As measured by Folin-Ciocalteu method, hydrolyzed lemon-yellow sorghum extract SC748-H contained higher total phenols than red sorghum 99LGWO50 extract (186.7 versus 103 mg gallic acid equivalent/g extract, Table 14). However, 99LGWO50 extract had 4 times higher amount of flavones than the SC748-H extract (4.84 versus 1.12 mg/g, Table 10). On the other hand, 99LGWO50 extract contained less than one tenth of flavanones in SC748-H extract (2.61 versus 28.1 mg/g). This compositional differences suggest that even though both flavones and flavanones are ERβ agonists in YAMC cells, the flavones (dominant in 99LGWO50 extract) possess higher estrogenic potency than flavanones (dominant in SC748-H extract). This also indicates that the low content of flavanones and absence of flavones in red TX2911 sorghum extract (Table 14) were probably why TX2911 extract did not show estrogenic activity. Thus we concluded that flavones are the most potent estrogenic compounds in sorghum.

Interactive Effects of Flavones and Flavanones on Estrogenic Activity

To further determine relative potency and potential interactive effects of flavones and flavanones on ERβ activation, apigenin and naringenin were tested for their growth inhibition effect in YAMC cells. Apigenin inhibited YAMC cell growth similarly to 1 nM estradiol at 1 μM; naringenin had a similar effect at 10 μM (Figure 23A). Administration of ICI reversed the growth inhibitory effect of apigenin (1 μM) and naringenin (10 μM) to the level of control (Figure 23B), which suggested their growth

inhibition effect was mediated through ERβ. The difference in relative estrogenic potency of apigenin and naringenin on ERβ was consistent with other reporter assays targeting ERβ signaling (*31*). The ERβ activation by naringenin was previously reported in cell models transfected with ERβ (*31, 181*). These studies may under- or over-estimate the effects of phytoestrogens in physiological system. This is the first report that suggests naringenin activates ERβ in a model naturally expressing ERβ as the predominant ER. As observed in sorghum extracts, 99LGWO extract high in flavones had higher potency in estrogenic activity than SC748-H extract high in flavanones. The differences in relative potency of apigenin (a flavone) and naringenin (a flavanone) may partly explain this observation.

Both apigenin and naringenin acted as ER agonists in YAMC cells. However, the concentrations at which the pure compounds were active were much higher than the concentrations of flavones or flavanones in sorghum extracts which showed estrogenic activity in YAMC cells (Table 15). One drawback of using pure compounds to investigate the biological effects of a complex system, such as foods, is that it ignores the possible synergism or interaction among different compounds present in the complex system. We believe the presence of both ERβ agonists, flavones and flavanones, as well as other phenolic compounds in sorghum extracts, may enhance the estrogenic activity of putative ERβ agonists in YAMC cells. To investigate this possibility, a combination of apigenin and naringenin were tested at different concentrations in the YAMC cell model.

Table 15. Concentrations (μM) of flavones and monomeric flavanones in sorghum extracts as tested in cell models (5 μg/mL, except White sorghum extract at 10 μg/mL).

	Flavones (μM)	Flavanones (μM)
TX2911 (red)	0	0.010
White	0.090	0
TX430 (black)	0.035	0
99LGWO50 (red)	0.091	0.048
SC748-H (hydrolyzed lemon-yellow)	0.019	0.505

Values are derived from means of compounds in Table 8 and Table 10.

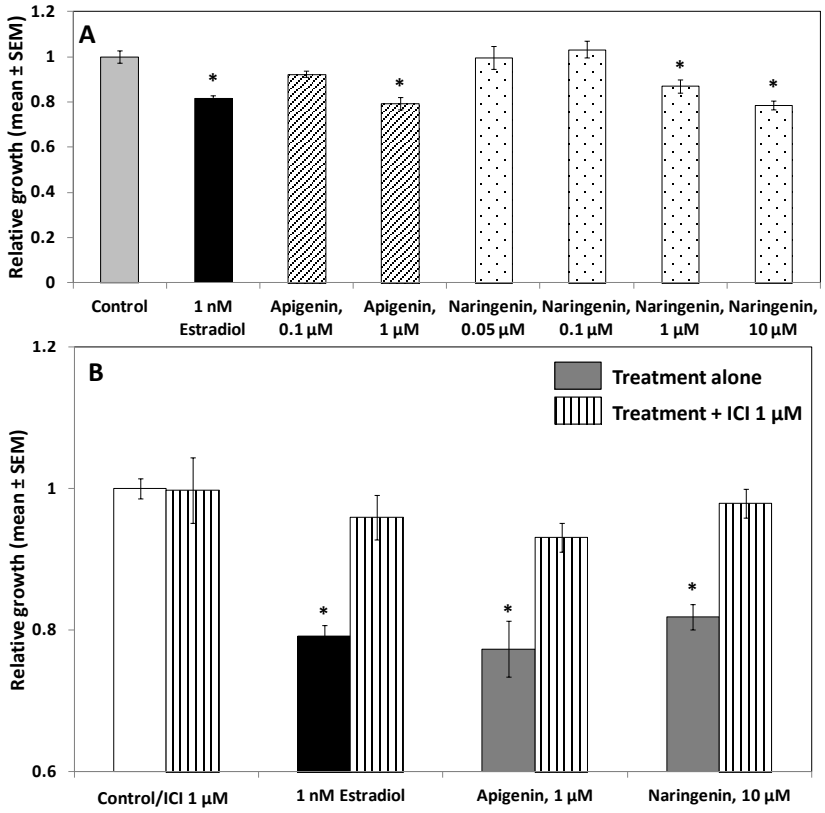


Figure 23. The effect of (A) apigenin (0.1 and 1 μM) and naringenin (0.05-10 μM); (B) co-treatment of 1 μM ICI 182, 780 (estrogen receptor antagonist) with apigenin (1 μM) and naringenin (10 μM) on the growth of non-malignant young adult mouse colonocytes (YAMCs). Data are expressed as mean ± SEM from three separate experiments. Dunnett's t test was used to compare the least square means of each treatment with corresponding control post a one-way ANOVA analysis. Treatments with an asterisk indicate significant difference from the control (DMSO) ($p < 0.05$).

When different levels of naringenin (0.1-10 μM) were added to the optimal concentration of apigenin (1 μM), the results showed that sub-optimal concentrations of naringenin (0.1 and 1 μM) did not enhance the growth inhibitory effect of 1 μM apigenin (Figure 24A). However the optimal concentration of naringenin (10 μM) surprisingly eliminated the growth inhibitory effect of 1 μM apigenin (Figure 24A). This antagonistic effect was unexpected. A plausible explanation may be competitive binding for ERβ. The ligand-binding domain of ERβ produces a more hydrophobic binding pocket when bound to flavonoid agonists (*182*). Apigenin is slightly more hydrophobic than naringenin, which may contribute to its stronger ERβ affinity and estrogenic potency than naringenin. When there are significantly more molecules of naringenin than apigenin, it is possible that naringenin, the less favored agonist, prevents the binding of apigenin to ERβ. However, this competition may also prevent naringenin from binding to ERβ. As a result, neither compound can sufficiently bind to ERβ and produce significant changes. Another plausible explanation is that naringenin could interfere with the regular genomic ER activation, such as dimerization of ER and recruitment of co-activators, after apigenin binds to ERβ. Changes in genomic ER activation after co-administration of apigenin and naringenin need to be monitored to better understand the interaction. The loss of estrogenic activity after combining optimal levels of both apigenin and naringenin may suggest that pure compounds at high levels, such as can be obtained through dietary supplements, could bring undesirable effects on intended bioactivity.

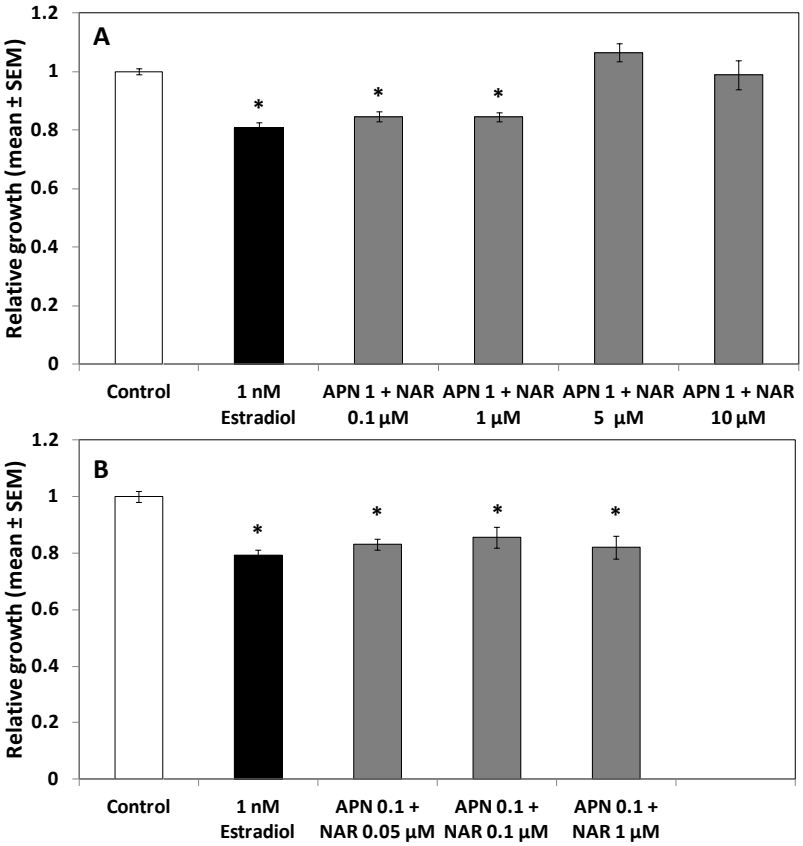


Figure 24. The effect of (A) apigenin (APN 1 µM) co-treated with naringenin (NAR 0.1-10 µM); (B) apigenin (0.1 µM) co-treated with naringenin (0.05-1 µM) on the growth of non-malignant young adult mouse colonocytes (YAMCs). Data are expressed as mean ± SEM from three separate experiments. Dunnett's t test was used to compare the least square means of each treatment with corresponding control post a one-way ANOVA analysis. Treatments with an asterisk indicate significant difference from the control (DMSO) ($p < 0.05$).

Interestingly, combination of sub-optimal concentrations of apigenin (0.1 µM) and naringenin (0.05, 0.1, and 1 µM) enhanced the growth inhibition of YAMC cells, which suggested enhanced ER activation (Figure 24B). For example, apigenin at 0.1 µM and naringenin at 0.05 µM together showed activity similar to 1 nM estradiol, which was 1/10 and 1/200 of their corresponding optimum concentrations. This observation is

interesting because, firstly it indicates the activity of target compounds in complex systems may be much higher than what can be examined or modeled using pure compounds. The data also suggest that in general, the concentrations of flavones and flavanones in sorghum extracts are adequate to activate ER in YAMC cells (Table 15). The red TX2911 sorghum extract which was not estrogenic in either cell models predominantly expressing ERα or ERβ was the only sorghum extract tested that did not contain any flavones. It only contained very low levels of flavanones, primarily naringenin-7-*O*-galactoside. In general it appears that presence of flavones is a good predictor of estrogenic potential of sorghum.

Conclusions

We demonstrated that sorghum phenolic extracts show estrogenic activity in cells that primarily express ERα (MCF-7) or ERβ (YAMCs). Composition of phenolic compounds plays an important role in the estrogenic activity of sorghum varieties. Flavones are the most important phenolic compounds in predicting estrogenic potential of sorghum. Flavanone aglycones also showed estrogenic potential but flavanone glycosides, the predominant form of flavanones in sorghum, were less effective. The active concentrations of flavones and flavanones in sorghum extracts were much lower than what was predicted by pure compounds. This was likely due to additive or synergistic effects of the complex mixture of phenolic compounds present in sorghum. The observed estrogenic properties of sorghum phenolics in non-malignant epithelial colonocytes may imply protection against colon carcinogenesis at concentrations

achievable through diet. This information is important in improving the understanding of how sorghum phenolics influence prevention of estrogen dependent cancers. This information could also be used by plant breeders to selectively breed sorghum varieties for specific health benefits. Follow up *in vivo* studies in mice are necessary to confirm this protection of estrogenic sorghum extracts against colon carcinogenesis and understand their bioavailability. Findings of this study also imply that modeling bioactivity with pure compounds may be misleading, as it does not reflect the true holistic effect of such compounds in a complex system.

CHAPTER IV

SORGHUM PHENOLIC EXTRACTS PREVENT AZOXYMETHANE INDUCED COLON CARCINOGENESIS IN OVARIECTOMIZED MICE

Introduction

Observational studies have found lower incidence of colon cancer in females compared with males (*8, 9*), and also in premenopausal women compared with post-menopausal women (*10*). Long term administration of oral contraceptives has been associated with reduced colon cancer incidence in women (*102*). Multiple observational studies showed approximately 30% reduction in relative risk for colon cancer in postmenopausal women who received hormone or estrogen replacement therapy compared with those who did not (*103-105*). These evidence suggest that estrogen plays a protective role in the development of colon cancer. It has been proposed that protective effects of estrogen are mediated via estrogen receptor-β (ERβ), the predominant estrogen receptor in the colon. These activities such as induction of apoptosis (*11*) and tumor suppressor genes (*12*) have been demonstrated in azoxymethane (AOM) induced colon carcinogenesis model of ovariectomized mice.

We have shown in previous chapter that phenolic extracts of sorghum are estrogenic in non-malignant colonocytes and are capable of inducing apoptosis in damaged colon epithelial cells. Flavones, especially apigenin, were identified as the most potent estrogenic compounds in sorghum. The potential of these sorghum phenolics with inherent estrogenic activity to prevent colon cancer needs further

investigation. The goal of this study is to determine the colon cancer chemopreventive potential of sorghum phenolic extracts with estrogenic activity. The hypothesis is that the estrogen-like activities of sorghum phenolic extracts could prevent formation of premalignant lesion (aberrant crypt foci, ACF) induced by AOM in colon of ovariectomized mice. The purpose of utilizing ovariectomized mice model is to eliminate the protective effect from edocrine estrogens (*11*). Two sorghum extracts, white and black, were selected in this study based on phenolic composition. Besides determination of the number of ACF as the biomarker for the onset of colon carcinogenesis, physiological changes (proportion of proliferative and apoptotic colonocytes) were also measured in fixed distal colon sections by immunohistochemistry to identify possible protective mechanisms induced by sorghum phenolic extracts.

Materials and Methods

Materials

Treatments: freeze-dried phenolic extracts of white and black sorghum. Two non-tannin sorghum grains were selected based on the results from Chapter 3: a white variety (ATXArg-1/RTX436) and a black variety (A05029/TX3362). The two sorghum grains were of the same genetic background as White (ATX635×RTX436) and TX430 (black) used in Chapter 3, respectively, and had similar overall composition of phenolic compounds. The grains were kindly provided by Dr. W. L. Rooney in the Department of

Soil & Crop Sciences at Texas A&M University. The grains were decorticated to collect bran (15% yield) for extraction. The bran was first defatted using hexanes (1:2 w/v ratio for 2 hrs) then extracted with 70% aqueous acetone 3 times (1:4 ratio, 100 g portion, 2 hrs then 1 hr and 1 hr duration of extraction); finally extracted with 0.01% formic acid in methanol (1:4 ratio) 2 times for 1 hr each time. The supernatant from all extractions was collected by filtering through a No.1 filter paper (VWR, Radnor, PA) and combined. Organic solvent was removed by rotary evaporation (Rotovap, Büchi, Flawil, Switzerland) under vacuum at 40 °C and aqueous fraction was freeze-dried to obtain powder extracts.

Animals (*11*). Wild type female c57BL6/J mice of 6-7 weeks old were purchased from Charles River Laboratories (Wilmington, MA). All animals were housed at the Laboratory Animal Resources and Research facility at Texas A&M University. All the handling and treatment procedures were performed under an animal use protocol approved by the Institutional Animal Care and Use committee at Texas A&M University. Ovariectomy was performed on all mice after 1 week of receiving. At the time of surgery, mice were randomly divided into treatment groups and started treatment diet (n=15, Table 16). One group (treatment) received estradiol treatment by implanting an estradiol pellet (0.5 mg estradiol 19.5 mg cholesterol, both from Sigma-Aldrich, St. Louis, MO) under skin at the back of the neck. All other groups (i.e. control and sorghum diet treatment groups) were implanted with cholesterol pellet (20 mg

cholesterol) (Table 16). The pellets were replenished at week 10 to ensure steady supply of estradiol (Figure 25).

Diet. Mice were fed with phytoestrogen free and isocaloric base powdered diet (AIN-76; TestDiet, Purina Mills LLC., St. Louis, MO). Mice were given 3 weeks after surgery to recover and adapt to powdered diet. Mice had access to food and water *ad libitum* during the study. Diet was replenished daily. White sorghum extract was added to base diet at 1% (w/w) level, and black sorghum extract was added at 1% and 1.5%, respectively (Table 16). The level of sorghum extract substituted in each diet was designed to achieve a circulating level that is at least equivalent to the concentrations used in YAMC cell culture studies. The target compounds chosen were flavones and the oral absorption rate of flavones was estimated based on literature reports (*183*). Based on the fact that white sorghum extract had higher level of apigenin, the compound believed to be the most potent ERβ agonist in the sorghum extracts, a treatment with higher level of black sorghum extract was used (1.5% in diet) to determine if a dose-response would exist.

Table 16. The experimental design of sorghum phenolic extracts in preventing azoxymethane (AOM) induced colon carcinogenesis study.

Treatment group	Diet	Pellet type	Functions
1	Control diet	Cholesterol pellet	Control
2	White 1.0%	Cholesterol pellet	Treatment
3	Black 1.0%	Cholesterol pellet	Treatment
4	Black 1.5%	Cholesterol pellet	Treatment
5	Control diet	Estradiol pellet	Positive control

Methods

Food intake. In order to monitor the consumption of diet across groups and different time points during the study, mice were single housed and fed with corresponding diets for 24 hr to record food intake. The weight difference of diet before and after the 24 hr period was the amount of food each animal consumed. Food intake was recorded 3 times during the study. Feces of each mouse were collected at baseline (before start of treatment diet), each food intake point, and the day of sacrifice (Figure 25). Body weight was recorded on a weekly basis.

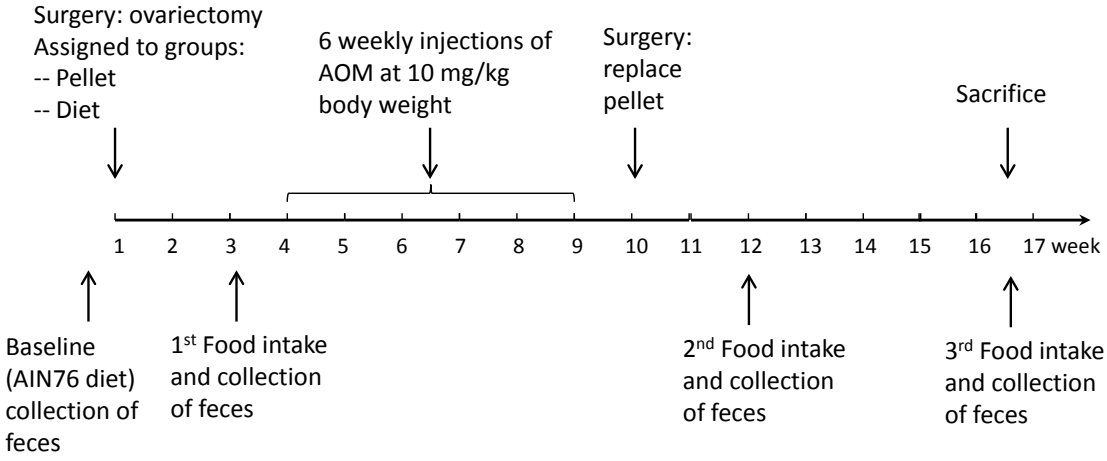


Figure 25. Timeline of the effect of sorghum phenolic extracts on preventing azoxymethane (AOM) induced colon carcinogenesis study.

Induction of preneoplastic lesions in the colon (*11*). Azoxymethane (AOM, Sigma-Aldrich, St. Louis, MO) was injected at 10 mg/ kg body weight weekly for 6 weeks,

starting from the 4th week after surgery (*184*); 7 weeks after the last AOM injection, mice were sacrificed (Figure 25). The AOM stock solution was dissolved in saline solution at 3.33 mg/mL.

Serum and tissue collection. At time of sacrifice, blood was collected in EDTA (0.3 M) containing microcentrifuge tubes by cardiac puncture method, and plasma was separated from whole blood by centrifuge at 5000 X *g* for 15 minutes at 4 °C. Plasma was aliquoted in 0.1 mL portions and stored at -20 °C till use. Plasma level of estradiol was determined by an ELISA kit (Estradiol EIA kit, Cayman Chemical Co., Ann Arbor, MI) following the instructions of the manufacturer. Uteri were collected and put in phosphate buffer saline (PBS) to record wet weights. Colons were collected, recorded length, then flattened between two flat glass boards and fixed in 70% ethanol. One cm tissue from both proximal/distal ends of the colon was resected, cassetted and fixed in 4% paraformaldehyde (4 hrs). Fixed distal end colon tissue was paraffin embedded, sectioned into 4 μm thickness and mounted to glass slides for immunohistochemical analysis.

Evaluation of aberrant crypt foci (ACF). Colons (except 1 cm tissue from proximal/distal ends) were fixed with 70% ethanol. At the time of experiment, the colons were stained with 0.5% methylene blue solution to count the total numbers of ACF and high multiplicity ACF (consisting of 3 or more individual ACFs in one cluster) in each colon using a bright field microscope mounted with a camera.

BrdU proliferation assay. Proportions of proliferative colonic crypt cells were determined by the ability of cells to incorporate bromo labeled nucleotide (5-bromo-2′-deoxyuridine, BrdU, a thymidine analog, Sigma-Aldrich, St. Louis, MO) into DNA synthesis. Two hours before sacrifice, the mice were injected with 30 mg BrdU/kg body weight (4 mg/mL stock solution in PBS) for further immunohistochemical determination in fixed distal colon sections. Tissue sections were first deparaffinized by 2 washes of xylene then rehydrated with sequential washes in 100%, 95%, 70% ethanol and finally in distilled water. Endogenous peroxidase activity was quenched by incubating tissues with 3% hydrogen peroxide in 100% methanol for 30 min at room temperature. After three washes with distilled water, tissues were prepared for antigen retrieval by heating for 20 min in a buffer solution containing 0.18 mM citric acid and 0.82 mM sodium citrate. Slides were washed with PBS buffer after cooling to room temperature. In order to eliminate non-specific binding, the tissues were blocked with 3% fetal bovine serum in PBS for 20 min before the application of primary antibody. Primary antibody (mouse-anti-BrdU, Roche USA, Indianapolis, IN) was diluted 20 times with PBS and applied to each tissue section, then incubated in a humidified chamber overnight at 4 °C. Next, tissues were washed with distilled water and applied with secondary antibody (200 times diluted with PBS, goat-anti-mouse horseradish peroxidase conjugated antibody, Abcam, Cambridge, MA) then incubated in a humidified chamber for 1 hr at room temperature. Following three washes with PBS, tissues were incubated with 1% diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, MO) in PBS for 10 min for secondary antibody color development. The tissues were then counterstained with 7g/L Harris modified

Hematoxylin solution (Sigma-Aldrich, St. Louis, MO) for 15 seconds, and washed with distilled water. Lastly, tissues were dehydrated with sequential washes in 70%, 95%, 100% ethanol and xylene, then coverslipped with Permount (Thermo Fisher Scientific, Pittsburg, PA).

TUNEL assay. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays were used to determine the apoptotic cells in fixed distal colon sections. An ApopTag peroxidase in situ Apoptosis Detection Kit (Millipore, Billerica, MA) was used following manufacturer's instructions with some modifications. Tissue sections were first deparaffinized by 3 washes of xylene then rehydrated with sequential washes in 100%, 95%, 70% ethanol and PBS. Tissues were treated with protein kinase K (10 µg/mL in PBS) for 3 min at 37 °C. Endogenous peroxidase activity was quenched by incubating tissues with 0.3% hydrogen peroxide in 100% methanol for 30 min at room temperature. After washing with distilled water and PBS, the tissues were equilibrated with equilibration buffer for 20 seconds at room temperature then applied with TDT enzyme in reaction buffer for 1 hr at 37 °C. Tissues treated with reaction buffer (no TDT enzyme) served as the negative stain control. The reaction was stopped by incubating the tissues with stop wash for 10 min at room temperature. After wash with PBS, tissues were applied with anti-digoxigenin and incubated for 30 min at room temperature. Followed by PBS wash, color was developed by incubating tissues with 0.5% DAB in PBS for 20 seconds, and was counter stained with 0.5% methyl green in pH 4.0 sodium

acetate solution for 20 seconds. Lastly, tissues were dehydrated with sequential washes in 70%, 95%, 100% ethanol and xylene, then coverslipped with Permount.

Tissue analysis. For both proliferation and TUNEL assays, 20 well-oriented intact crypts were analyzed for each animal. Crypts were divided into three equal sessions along the sides, as top, middle, bottom, respectively. The percentage of positive cells was counted in each session for each crypt of each animal, by dividing number of positive cells by total number of cells in that session. The total percentage of positive cells was summed from all three sessions. The percentages of proliferative and apoptotic cells in total and in each session were averaged for each animal and compared across different treatments.

Statistical analysis. The data obtained in this Chapter were data from counts. In order to compensate for unequal variance and lack of normality, Poisson regression model (with natural logarithm transformation) was utilized to analyze the difference among groups by a generalized linear model procedure of SAS (proc GENMOD). The t test was used to compare least square means of transformed data to determine treatment differences. All statistical analysis was performed with SAS 9.2 (Cary, NC). Data were still presented in their original untransformed form for interpretation purposes. Data obtained from animal #30, #22, #24, and #71 were eliminated in all analysis due to abnormal physiological conditions at time of sacrifice. Animal #30 had blood spots in the liver and abnormal serum color; animals #22, #24, and #71 had swollen kidney problems and/or necrosis in the intestine. The sample size of each group used for analysis was 14, 11, 14,

15, and 15 for control, estradiol, White sorghum 1%, Black sorghum 1%, and Black sorghum 1.5%, respectively.

Results and Discussion

Phenolic Composition and Antioxidant Capacity of the Sorghum Extracts

The major phenolic compounds in the two sorghum extracts were identified and quantified according to the methods described in Chapter 3. The two sorghum varieties shared the same genetic background with the White (ATX635×RTX436) and TX430 (black) sorghum respectively, hence had similar compositions. Table 17 summarizes the contents of major phenolic compounds in these two sorghum extracts used. Flavone content in white sorghum extract was 4.31 mg/g extract, most were apigenin (3.93 mg/g). The black sorghum extract contained 2.26 mg/g flavones, with 0.57 mg/g luteolin and derivatives.

Table 17. Contents[a] (mg/g extract) of major phenolic compounds in white and black sorghum extracts used in the animal study.

Compound	White	Black
Total phenols[b] (mg gallic acid equivalent/g)	**28.9 ± 1.54**	**268 ± 25.1**
2-*O*-Caffeoulglycerol-*O*-glucoside	Trace	ND
1-*O*-Caffeoulglycerol-*O*-glucoside	0.543 ± 0.047	ND
2-*O*-Caffeoulglycerol	0.066 ± 0.004	0.064 ± 0.014
1-*O*-Caffeoulglycerol	0.473 ± 0.016	0.110 ± 0.027
Caffeic acid	0.217 ± 0.009	0.084 ± 0.016
Ferulic acid-*O*-galactoside	0.210 ± 0.014	ND
Ferulic acid-*O*-glucoside	ND	ND
Ferulic acid	ND	ND
Dicaffeoylglycerol	0.216 ± 0.014	ND
Dicaffeoylglycerol	3.23 ± 0.17	ND
Dicaffeoylglycerol	Trace	ND
p-Coumaroyl-caffeoyl-glycerol	1.39 ± 0.018	ND
p-Coumaroyl-feruloyl-glycerol	ND	1.41 ± 0.045
Diferuloylglycerol	ND	0.916 ± 0.074
Feruloyl-caffeoyl-glycerol	1.15 ± 0.057	ND
Total phenolic acids and esters[c]	**7.50 ± 0.32**	**2.59 ± 0.17**
4'-OMe-Luteolin-7-*O*-rutinoside	ND	0.181 ± 0.040
Luteolin	0.197 ± 0.017	0.389 ± 0.018
6-*C*-glucosyl-8-*C*-arabinosyl-Apigenin	Trace	ND
Apigenin	3.93 ± 0.33	0.264 ± 0.033
3'-methoxy-4', 5', 5, 7-tetrahydroxyflavone	ND	0.362 ± 0.010
Tricin	0.178 ± 0.009	0.612 ± 0.002
3', 5'-methoxy-4', 5', 6, 7-tetrahydroxyflavone	ND	0.451 ± 0.008
Total flavones[c]	**4.31 ± 0.36**	**2.26 ± 0.010**
Luteolinidin (LUT)	ND	1.47 ± 0.01
Apigeninidin (APG)	ND	1.26 ± 0.03
7-OMe-LUT	ND	0.273 ± 0.04
7-OMe-APG	ND	0.276 ± 0.02
Total 3-deoxyanthocyanins[c]	**ND**	**3.27 ± 0.11**

[a]All values are expressed as mean ± SD of two separate runs, as quantified by HPLC with appropriate standards. [b]Measured by Folin-Ciocalteu method. [c]Sum of major peaks identified and quantified by HPLC.

The white sorghum extract also had higher concentrations of phenolic acids (7.50 mg/g) than the black sorghum extract (2.59 mg/g). On the other hand, the black sorghum extract contained 3-deoxyanthocyanins (3.27 mg/g) while the white sorghum extract did not. Flavanones, such as naringenin or eriodictyol and their derivatives, were not detected in these two sorghum extracts. Overall, the black sorghum extract had much higher total phenols (268 mg gallic acid equivalent/g extract measured by Folin-Ciocalteu method) than the white sorghum extract (28.9 mg gallic acid equivalent/g extract).

Effect of Sorghum Extracts on Animal Growth and Uteri Development

Supplementation of sorghum phenolic extracts to basal diet did not affect the growth of the animals, or the trend of weight gain (data not shown). The groups did not show significant differences in each food intake assays, final body weight, or the length of colon (data not shown).

The estradiol group had 1.43 ± 0.23 nmol/L (mean ± SEM) estradiol in plasma, whereas the plasma estradiol level in all other animals implanted with a cholesterol pellet was below the detection limit (0.014 nmol/L). Estradiol group also had significantly higher wet weight of uteri than control group (0.5 ± 0.15 g versus 0.055 ± 0.01 g, $p < 0.05$), which indicated that estradiol successfully released into circulation. The wet weight of uteri in all sorghum extract fed groups did not show differences relative to control, and were 0.041 ± 0.004, 0.047 ± 0.007, and 0.044 ± 0.004 g, for groups fed with white extract 1%, black extract 1% and 1.5%, respectively.

Estradiol is essential to support the normal development of uteri. At the beginning of the study, the mice were just coming to sex maturation and ovariectomy would stop the development of uteri. Estradiol pellet released estradiol to maintain regular development of uteri in the estradiol group. However, the sorghum extracts did not maintain development of uteri, which indicated at the level of sorghum extracts fed, the compounds in sorghum were not effective ERα agonists or were not absorbed to a critical concentration to maintain development of uteri. Au et al. (*185*) reported that female mice which were fed with up to 0.1% apigenin in the diet had no significantly different uterine weights compared with control non-apigenin fed animals. This concentration of apigenin (100 mg/100g diet) was roughly 25 times higher than what was present in the white sorghum (1%) diet (3.93 mg/100g diet). Apigenin was suggested as more selective for ERβ than ERα (*31*), thus the limited uteri growth capacity does not necessarily mean no ERβ mediated activity would be expected.

Effect of Sorghum Extracts on Formation of Azoxymethane Induced Colonic Premalignant Lesion (Aberrant Crypt Foci) in Ovariectomized Mice

The effect of sorghum extracts on formation of azoxymethane (AOM) induced colonic premalignant lesion (aberrant crypt foci, ACF) in ovariectomized mice model is shown in Figure 26. Compared with control group, white sorghum extract, fed at the level of 1% in diet, significantly reduced the total number of ACF in the colons, by 39.3% ($p < 0.001$). Black sorghum extract, fed at the same level (1%), also significantly reduced the total number of ACF, but to a smaller extent (by 14.7 %, $p < 0.044$). The

higher level of black sorghum extract, 1.5%, showed a mild 9.3% reduction of total number of ACF but was not significantly different from the control ($p = 0.202$). Surprisingly, the estradiol group had only 6.1% reduction of total ACF ($p = 0.448$), which was not different from control (Figure 26A).

The effect of sorghum extracts on formation of high multiplicity ACF (≥ 3 single ACFs in one cluster) was not the same as total ACF. In general none of the treatments showed a significant effect relative to control (Figure 26B). Compared with control group, white sorghum extract at 1% level, reduced 26.4% high multiplicity ACF, but the effect was not statistically significant ($p = 0.146$); estradiol group reduced high multiplicity ACF by 16.0%, which was not statistically significant as well ($p = 0.425$).

The capacity of a treatment to reduce numbers of ACF formed in distal colons suggests protection against AOM induced colon carcinogenesis. The white sorghum extract fed at 1% level showed protective action in reducing both total numbers of ACF and numbers of high multiplicity ACF. However, the black sorghum fed groups did not show consistent trends, and only suggested a mild protective effect. The differences of protective effects observed in the animal study could be attributed to the phenolic profiles of these two extracts. Our cell culture data suggested that apigenin was the most potent estrogenic compound in sorghum extracts (Chapter 3). White sorghum extract had a much higher concentration of apigenin (3.93 mg/g) than black sorghum extract (0.264 mg/g), which indicates the protective effect of white sorghum, was probably related to its high content of apigenin. Interestingly, black sorghum extract had much higher total phenols than white sorghum extract (Table 17). This again indicates that phenolic

composition may be a better predictor of bioactivity than total phenols content. Also, the presence of other possibly non-estrogenic phenolic compounds in the black sorghum extract at such high levels could have interfered with the activity of the estrogenic components.

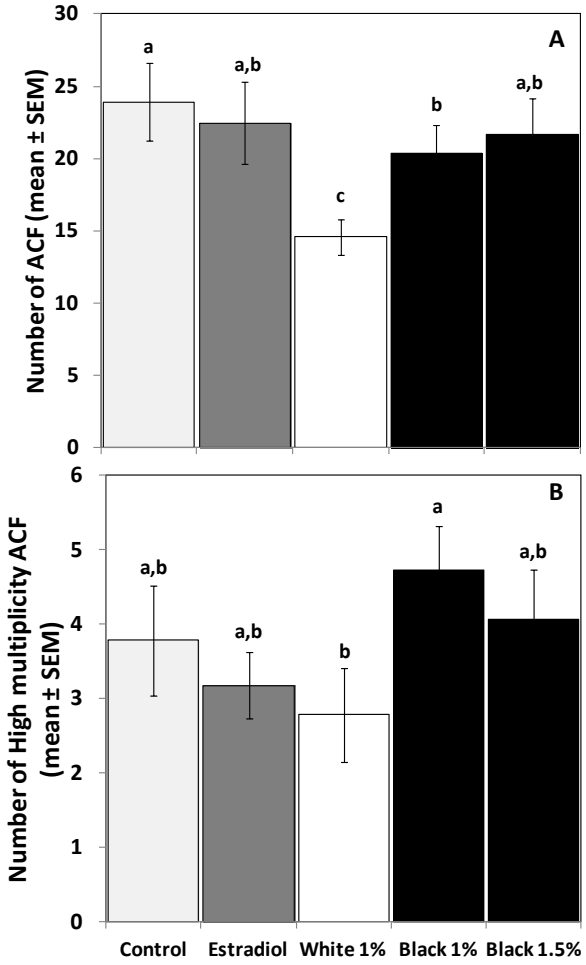


Figure 26. The effect of crude sorghum extracts, white (1%) and black (1% and 1.5%), fed in the diet on total numbers of (A) aberrant crypt foci (ACF) and (B) high multiplicity ACF identified in the distal colons of ovariectomized mice injected with azoxymethane (AOM). Data are expressed as numbers identified per animal (mean ± SEM). Data with the same letter do not differ ($p < 0.05$).

Effect of Sorghum Extracts on Proliferation and Apoptosis of Colonocytes

Induction of apoptosis is proposed as a protective mechanism against AOM induced colon carcinogenesis (*11*). In order to explore if changes in apoptosis or proliferation in colonocytes can be mechanisms of reduced total numbers of ACF in colons, percent of apoptotic and proliferative cells in 20 well-oriented crypts per animal were determined.

No statistical differences were observed in average percent of apoptotic cells per animal, but some trends were observed (Figure 27). Estradiol group had a 38.7% increase in proportion of apoptotic cells overall, which may contribute to its mild effect on total numbers of ACF and high multiplicity ACF (Figure 26B). Black sorghum fed at 1.5% level also had a 27.0% increase in apoptotic cells. The white sorghum and black sorghum extracts fed at 1% did not show any increase of apoptotic cells and actually showed a slight decrease (Figure 27).

Weige et al. (*11*) reported that estradiol treatment (2 mg estradiol 18 mg cholesterol pellet) reduced the formation of total numbers of ACF by approximately 50%, and induced 5.7-fold increase in apoptotic cells in the same mice model (AOM induced colon carcinogenesis model of ovariectomized c57BL6/J mice). The plasma level of estradiol was around 5 nM. In a different study, Weige et al. (*186*) observed a similar effect on reduction of total number of ACF in wild type C57BL6/J mice, but with lower estradiol treatment (1 mg estradiol 19 mg cholesterol pellet) and plasma estradiol concentration (1.9 nM). However, the proportion of apoptotic colon cryptic cells was 45% higher in the estradiol group compared with that of control group.

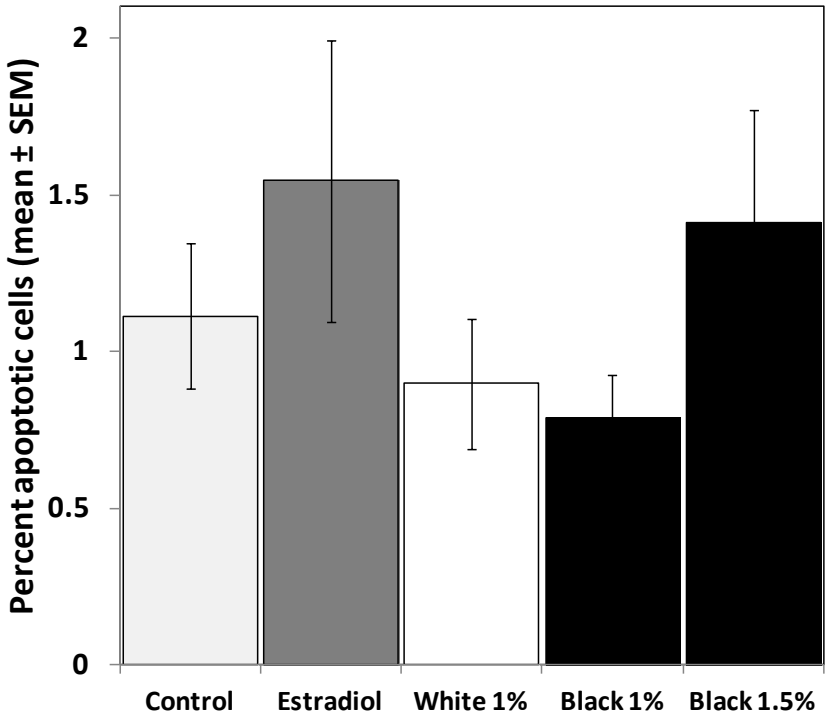


Figure 27. The effect of crude sorghum extracts, white (1%) and black (1% and 1.5%), fed in the diet on percent of apoptotic cells in one colonic crypt of the distal colon of ovariectomized mice injected with azoxymethane (AOM). Data are expressed as mean ± SEM from 20 well-oriented crypts per animal measured by TUNEL assay. The percent of apoptotic cells are obtained by dividing the number of apoptotic cells in each crypt, identified by immunohistochemical staining of distal colon sections, by the total number of cells in that crypt.

In order to better target a circulating estradiol concentration within the physiological range (1 nM), a pellet of 0.5 mg estradiol and 19.5 mg cholesterol was used in our study. This treatment produced around 1.4 nM estradiol in plasma of the mice. The limited inhibitory effect on formation of total ACF may be due to lower level of circulating estradiol in our study.

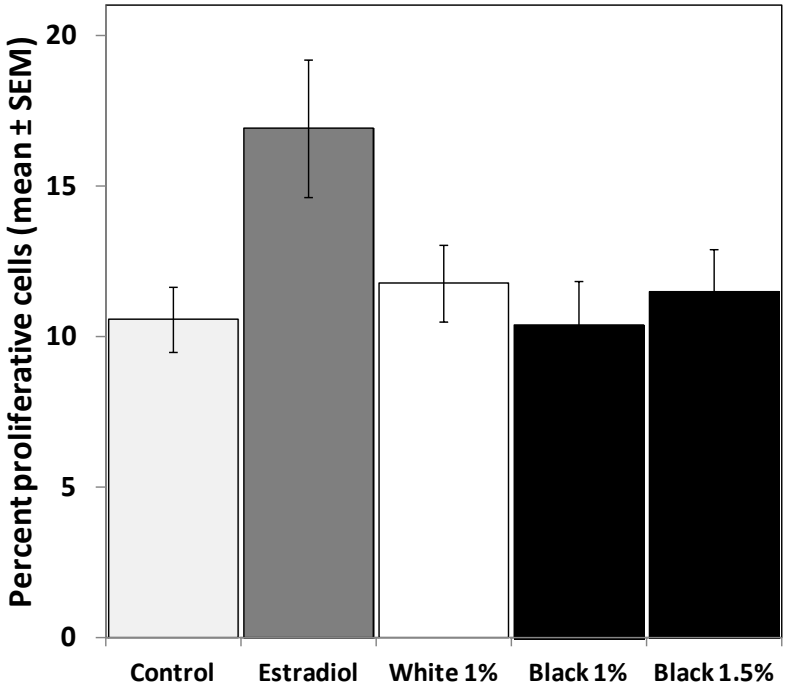


Figure 28. The effect of crude sorghum extracts, white (1%) and black (1% and 1.5%), fed in the diet on percent of proliferative cells in one colonic crypt of the distal colon of ovariectomized mice injected with azoxymethane (AOM). Data are expressed as mean ± SEM from 20 well-oriented crypts per animal measured by BrdU. The percent of proliferative cells are obtained by dividing the number of proliferative cells in each crypt, identified by BrdU immunohistochemical staining of distal colon sections, by the total number of cells in that crypt.

In terms of the percent of proliferative cells, estradiol treatment was the only group which showed an increase in proliferative cells (59.7% increase), but not statistically different than that of control ($p = 0.6705$) (Figure 28). Other treatments had similar percent of proliferative cells (10.4-11.8%) as control (10.6%) (Figure 28). Elevated proliferation of estradiol group may indicate faster cell change-over, which would have contributed to the limited inhibition against formation of ACF in estradiol group.

According to our results utilizing YAMC cells as a model for potential ERβ activation and estrogenic activity of sorghum phenolic extracts (Chapter 3), apigenin was suggested as the most potent estrogenic compound in sorghum. Apigenin was reported to induce reversible G2/M cell cycle arrest in different human adenocarcinoma cells, at the concentrations of 20-80 μM (*187*). The cell cycle arrest was believed to be related to reduced levels of $p34^{cdc2}$ and cyclin B1 proteins (*187*). The cell cycle arrest induced by apigenin was also reported to be *p53* dependent, and apigenin was able to stabilize *p53* proteins and induce apoptosis (*188, 189*). It is not clear whether these mechanisms were related to ER activation signaling, but induction of *p53* after activation of ER has been suggested as the mechanism by which estradiol prevents early colon carcinogenesis (*12*).

The protective effect of pure apigenin in reducing the AOM induced formation of premalignant lesions (ACF) in the colons of rodent models (approximately 50% reduction) was also reported by two independent studies, at the concentration of 0.1% apigenin added in the diet (*185, 190*). The proposed mechanisms were suppressing proliferation of colonocytes (*190*) and colonic ornithine decarboxylase activity (a rate limiting enzyme in polyamide biosynthesis) (*185*), but not induction of apoptosis (*190*). Interestingly, white sorghum extract fed at 1% level had a similar effect as previously reported for pure apigenin at 0.1% in diet (*185, 190*) in reducing total numbers of ACF. The finding was interesting and promising, considering that a crude extract was used and the level of apigenin in the diet (3.93 mg/100 g diet) was much lower than what was used in pure apigenin studies (100 mg/100 g diet). A plausible explanation could be that

other phenolic compounds in the white sorghum extracts enhanced the activity of apigenin or stabilized the apigenin targeted protective mechanisms, as suggested by the enhanced effect between apigenin and naringenin in YAMC cells in Chapter 3. However, compared with the studies utilizing pure apigenin, the white and black sorghum extracts did not induce significant changes in proliferative cryptic cells. Some other mechanisms need to be explored.

The black sorghum extract fed at 1% level in the diet was able to reduce the total number of ACF by 14.7% but not the high multiplicity ACF (Figure 26). The black sorghum extract contained 0.264 mg/g apigenin and a total of 2.26 mg/g flavones, mainly luteolin and methoxylated flavones (Table 17). Compared with the white sorghum extract, black sorghum extract had roughly half of the flavones present in white extract (2.26 versus 4.31 mg/g), which could partly explain the lower ACF reduction effect of black sorghum extract. Surprisingly, black sorghum extract fed at 1.5% level did not reduce the number of ACF significantly, compared with the effect observed for 1% black sorghum extract. The relatively higher levels of non-estrogenic phenolic compounds in the black sorghum extract may have interfered with the estrogenic properties of flavones. In Chapter 3, using the YAMC cell model, we observed that higher levels of naringenin antagonized the estrogenic activity of apigenin. The impact of higher levels of 3-deoxyanthocyanins in black sorghum on estrogenic activity of flavones deserve further investigation.

Similar to white sorghum extract, black sorghum extracts fed at 1% and 1.5% in the diet did not change the percentage of proliferative colon cryptic cells (Figure 28).

However, a trend towards induction of apoptosis of black extract at 1.5% was observed (27% increase, Figure 27). Thus more detailed studies should be carried out to understand the molecular mechanisms of the chemopreventive potential of white and black sorghum extracts against colon carcinogenesis in an ovariectomized mice model.

Future Studies

Our study showed that white and black sorghum extracts fed at 1% level in the diet were effective in reducing the total numbers of ACF formed in the colons of ovariectomized mice induced by AOM, by 39.3% and 14.7%, respectively. This suggests protection against colon carcinogenesis. Due to the fact that estradiol treatment did not significantly reduce total numbers of ACF and high multiplicity ACF, we can not conclude that these two sorghum extracts exerted protective effect through activities mediated via ERβ in the colon. Percent of apoptotic cells and proliferative cells did not suggest any clear mechanism for the protective effects observed. More detailed and specific mechanistic studies need to be conducted to explore the protective mechanisms of sorghum polyphenols. For example:

1) The effect of composition of sorghum phenolic compounds on preventing colon carcinogenesis *in vivo*. Other estrogenic sorghum extracts with various phenolic composition can be tested *in vivo*, such as the dark red 99LGWO50 and lemon-yellow SC748 sorghum varieties used in Chapter 3.

2) The effect of white and black sorghum extracts on key mutated genes and proteins related to colon carcinogenesis, such as *APC*, *COX-2*, and *p53*.

3) The effect of white and black sorghum extracts on enzymes and pathways related to metabolism and bioactivities of xenobiotics, such as cytochrome P450, cyclooxygenase-2, glutathione S-transferase, and Nrf-2 activation.

4) The roles of microbiota in the gut in facilitating the metabolism and absorption of sorghum phenolic compounds, as well as the possible roles of the metabolites from sorghum phenolics in participating in the protective activities against colon carcinogenesis.

CHAPTER V

THERMAL STABILITY OF SORGHUM 3-DEOXYANTHOCYANINS

Introduction

Consumption of sorghum has been associated with many health benefits; however, the research related to phenolics in sorghum is mostly conducted on raw grains. Sorghum grains are typically thermally processed to make food for humans. The thermal processes include boiling, steaming, baking, flaking and extrusion, among the others. Structural changes induced by thermal processing can impact food quality and bioactive properties of polyphenols. Understanding the extent of degradation of polyphenols, mechanisms involved, and degradation products is useful to determine the potential impact on processed foods, such as predicting quality changes over time and exploring methods to stabilize the polyphenols during thermal processing.

The common reactions of flavonoids induced by heat treatment include deglycosylation (*157*), breakage of ester bonds, generation of C6-C3-C6 structure fragments (e.g. protocatechuic acid) (*158-160*), as well as formation of neo-formation compounds (e.g. hydroxymethylfurfural, melanoidins) (*161*). These reactions contribute to flavor and color development during cooking (*161*), but the degraded and/or oxidized compounds may be involved in unfavorable reactions, such as oxidation (*162*). Heat treatment depolymerizes proanthocyanidins into lower molecular weight units (*91*), which are known to be more bioavailable. Some polyphenols, e.g. anthocyanins, can

polymerize during thermal processing, which can improve their color stability (*163, 164*).

Among the sorghum phenolics, the knowledge of thermal stability of 3-deoxyanthocyanins is very limited. These pigments possess unique characteristics as potential food colorants and chemopreventive agents. Compared to anthocyanins, 3-deoxyanthocyanins are not substituted at the C-3 position (Figure 3). Hence the 3-deoxyanthocyanins are more stable to pH changes (*27, 28*) and the bleaching effects of common food additives, like ascorbic acid and sulfites (*29*). In addition, they are more cytotoxic than their anthocyanidin counterparts (*30*). Therefore, determining their thermal stability will add valuable information to the potential of these pigments as bioactive food ingredients. In addition, knowing the degradation mechanisms is valuable in looking for strategies to stabilize the 3-deoxyanthocyanins during processing.

The pH of food matrix also influences color and stability of anthocyanins and 3-deoxyanthocyanins. When in solution, anthocyanins and 3-deoxyanthocyanins establish a complex equilibrium between colored flavylium cation, quinoidal base, colorless chalcone and carbinol pseudobase. The acidity (ideally pH 1-2) of the matrix favors the ionization reaction for the formation and stability of flavylium cation. Increase in pH brings the equilibrium towards more hydration of flavylium cation into carbinol pseudobase, which results in loss of characteristic color (*28, 191*). The pH of foods and food products varies from ~2.0 (lime juice) to ~7.9 (Graham crackers), the majority fall in between 3.0-6.0 (*192*). Most anthocyanins are not stable in this pH range while the 3-

deoxyanthocyanins remain relatively stable (*28, 86*). However, how pH affects stability of 3-deoxyanthocyanins during thermal processing is still not clear.

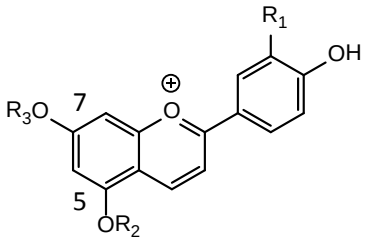
In order to evaluate thermal stability of 3-deoxyanthocyanins, the effect of heat treatment (121 °C, 30 min) at pHs 1-7, using different acidulants (HCl, formic acid and citric acid) were investigated. The severe time-temperature treatment was selected to ensure detectable changes (heat treatment at 95 °C for up to 2 hr did not result in meaningful changes, data not shown). Only acids were used in all solutions in order to eliminate the stabilizing effect from constituents of buffer systems. The 3 selected acidulants were aimed to compare the effect of mineral acid (HCl) with common organic acids (formic acid and citric acid). We hypothesize that type of acidulent and not pH alone, has an impact on stability of 3-deoxyanthocyanins.

Materials and Methods

Materials

Crude 3-deoxyanthocyanin pigments. Crude 3-deoxyanthocyanin extract was obtained from dried red sorghum leaf sheaths (Health Forever Products, Lagos, Nigeria). Acidified aqueous methanol (formic acid : water : methanol = 1 : 49 : 50) was used to extract the sample, with 2 hr shaking at room temperature. Solvent was removed to complete dryness under vacuum at 40 °C using a Multivapor system (Büchi, Flawil, Switzland).

3-Deoxyanthocyanidin compounds. In order to evaluate the effect of 3-deoxyanthocyanidin structure on its stability, major sorghum 3-deoxyanthocyanidins and methoxylated derivatives (Figure 29) were used in this study. Apigeninidin (APG), 5-*O*-methyl-apigeninidin (5-OMe-APG), 7-*O*-methyl-apigeninidin (7-OMe-APG), 5,7-*O*-methyl-apigeninidin (5,7-OMe-APG), luteolinidin (LUT), and 5,7-*O*-methyl-luteolinidin (5,7-OMe-LUT) were purchased from AlsaChim (Strasbourg, France). All pure compounds were synthetic and of at least 95% purity.



3-Deoxyanthocyanidins	R_1	R_2	R_3
Apigeninidin (APG)	H	H	H
7-Methoxyapigeninidin (7-OMe-APG)	H	H	CH_3
5-Methoxyapigeninidin (5-OMe-APG)	H	CH_3	H
5,7-Dimethoxyapigeninidin (5,7-OMe-APG)	H	CH_3	CH_3
Luteolinidin (LUT)	OH	H	H
5,7-Dimethoxyluteolinidin (5,7-OMe-LUT)	OH	CH_3	CH_3

Figure 29. 3-Deoxyanthocyanidins used in thermal stability study.

Methods

Sample solution preparation. Crude 3-deoxyanthocyanin extract (10 mg/mL) and pure 3-deoxyanthocyanidins (2 mg/mL, 5.94 - 6.84 mM) were reconstituted with HPLC grade

water (pH= 6.79) or acidified water of pHs 1-6 prepared with different acidulants (Table 18) in Pyrex test tubes with plastic stoppers. Samples were sonicated at 40% output energy for 30 s using a tip probe (6 mm diameter) sonicator (VibraCell 40, Sonics & Materials Inc., Danbury, CT). Immediately after sonication, 0.5 mL of each solution was transferred to a 2.0 mL microcentrifuge tube for thermal treatment.

Table 18. Design of 3-deoxyanthocyanin thermal stability experiment.

Factors	**Variables**
Pigments	Crude 3-deoxyanthocyanin extract Apigeninidin (APG) 7-OMe-Apigeninidin (7-OMe-APG) 5-O-Me-Apigeninidin (5-OMe-APG) 5,7-OMe-Apigeninidin (5,7-OMe-APG) Luteolinidin (LUT) 5,7-OMe-Luteolinidin (5,7-OMe-LUT)
Acidulants	Hydrocholoric acid (HCl) Formic acid Citric acid
pHs	1, 2, 3, 4, 5, 6, 7 (water)

Heat treatment. The heating experiment was carried out at 121 °C (0.13 MPa) for 30 minutes using an autoclave (SM-300, YAMATO Scientific Co., Ltd., Tokyo, Japan). At the end of heat treatment, the samples were put in ice to stop any further degradation reaction. An aliquot of each sample after 30 minutes equilibration in ice bath was taken to analyze color intensity change (UV-*vis* spectroscopy) and degradation profiles (HPLC and UPLC-MS). The experiment was performed in duplicates.

Change in color intensity. For crude 3-deoxyanthocyanin extract, 150 μL heated sample was diluted 20-fold with 1% HCl in methanol (pH=1.0). For pure 3-deoxyanthocyanidin compounds, 50 μL of heated sample was diluted 60-fold using the same solvent for analysis. The absorbance at their respective λ_{max} (A) was measured by a spectrophotometer (Shimadzu UV2450, Shimadzu Scientific Instruments, North America, Columbia, MD). A non-heat treated sample was diluted the same way and served as control (A_0). Color retention after heat treatment was calculated by comparing A with its respective control A_0.

HPLC analysis. To profile changes in 3-deoxyanthocyanins due to thermal treatment, 200 μL of treated crude 3-deoxyanthocyanins extract and 100 μL of treated pure 3-deoxyanthocyanidins were diluted 5-fold and 10-fold, respectively, with 4% formic acid in methanol (pH=1.0), then filtered through a 0.2 μm nylon membrane syringe filter. A non-heat treated sample was diluted and filtered the same way and served as control. An Agilent 1200 series HPLC system (Agilent Technologies, Santa Clara, CA) was used for this analysis. It included a quadratic pump (with degasser), an autosampler, a column compartment, and a diode array detector (DAD). A Luna C-18 column (150 mm × 4.6 mm, 5.0 μm, Phenomenex, Torrance, CA) was used to carry out the separation with a two solvent gradient: solvent A 1% formic acid in water and solvent B 1% formic acid in acetonitrile. The gradient based on solvent B was as follows: 0-3 min, 10%; 5 min, 18%; 10 min, 20%; 23 min, 26%; 25 min, 28%; 28 min, 40%; 30 min, 60%; 30-32 min, 60%; 34-40 min 10%. The column was kept at 40 °C during analysis and the flow rate was 1.0

mL/min. The injection volume was 20 μL for crude 3-deoxyanthocyanins extract and 5 μL for all pure 3-deoxyanthocyanidin compounds.

UPLC-ESI-MS analysis. To determine the structures of thermal degradation products, the 3-deoxyanthocyanidin samples used in this experiment were treated the same as for HPLC quantitative analysis. A Waters-ACQUITY UPLC/MS system (Waters Corp., Milford, MA) was used which consisted of a binary solvent manager, autosampler (sample manager), column heater, and photodiode array eλ detector (PDA) and interfaced with a tandem quadrupole (TQD) mass spectrometer equipped with an ESI source. The separation was performed on a Kinetex C18 column (150 mm × 2.10 mm, 2.6 μm, Phenomenex, Torrance, CA) at 40 °C with following gradient at 0.4 mL/min: Solvent A (0.05% formic acid in water), solvent B (acetonitrile), and the percentage of solvent B was 12-41% from 0-23.5 min, 41-75% from 23.5-25.5 min, 75% isocratic from 25.5-28.5 min, then 75-12% from 28.5-29.5 min, and 12% isocratic for 5 minutes to equilibrate the column. The injection volume was 1 μL. The monitoring wavelength for 3-deoxyanthocyanin pigments was at 485 nm; while thermal degradation products were monitored at 280 nm and 340 nm. Mass spectrometric data were acquired in positive mode for 3-deoxyanthocyanins, and in negative mode for degradation compounds. Empower 2 software (Waters Corp.) was used to acquire and analyze data. The MS scan was recorded in the range of 100−1000 Da. Nitrogen was used both as a drying gas and as nebulizing gas, while argon was used as the collision gas (AOC, Bryan, TX). The nitrogen gas flow conditions were 800 and 50 L/h for desolvation and

at the cone, respectively. The source block temperature and desolvation temperature were set at 150 and 400 °C, respectively. Optimization of ionization conditions was based on the intensity of the mass signals of protonated/deprotonated molecules and performed for each individual peak/compound detected. Mass parameters were optimized as follows: capillary voltage, 3.5/3.0 kV; and cone voltage, 60/30 V for positive/negative ionization, respectively. The MS/MS scan was optimized as follows: cone voltage of 45/(30-40) V and collision energy of 35/(15-30) V. Compound identification was based on matching UPLC retention profile, UV-*vis* spectra and MS data with authentic standards. Where standards were not available, compounds were identified based on the fragment patterns compared with reports in the literature.

Statistical analysis. Data were analyzed with one way analysis of variance (ANOVA) and treatment means were compared by Tukey's HSD test. Differences between two groups of samples were compared by Student's t-test. All statistical analysis was performed by SAS 9.2 (Cary, NC).

Results and Discussion

Thermal Stability of Crude 3-Deoxyanthocyanin Extract: Effect of pH and Acidulant

The dried leaf sheath from red sorghum has very intense red color. It is commonly used in West Africa as a source of dye for various food and non-food applications (*71*). The 3-deoxyanthocyanins in the crude extract comprised mostly of

apigeninidin (7.5 mg/g), 7-OMe-apigeninidin (0.51 mg/g), and luteolinidin (0.27 mg/g), which was similar to what was reported previously (*193*). It also contained dimeric 3-deoxyanthocyanidin pigments (*193*). Sorghum with red secondary plant color tends to accumulate mainly apigeninidin type pigments (*24, 25*). Besides 3-deoxyanthocyanins, the crude 3-deoxyanthocyanin extract also had a fair amount of apigenin (0.84 mg/g). Other phenolic compounds usually found in red sorghum, such as phenolic acids and flavanones, were detected in trace amounts.

The effect of pH and acidulants on color stability of crude 3-deoxyanthocyanin extract is shown in Figure 30. For HCl, color retained best in low pH conditions, i.e. pH 1 and 2, with less than 7% loss of absorbance. As expected, the color retention decreased with decreased acidity between pHs 3-6 (76-39%). Color retention in distilled water was 65%. For citric acid, the trend was similar to HCl, color retained best at pH 1. At pH 2 and 3, a drastic decrease was observed, with 71% and 45% color retained, respectively. Color retention at pHs 4-6 were generally similar and ranged from 45-56%. Interestingly, formic acid showed a different trend in low pH conditions. Color retention in pH 1 and pH 2 formic acid solutions was 61% and 48%, respectively, which were much lower compared with those of HCl and citric acid ($p < 0.05$). Color retention at pHs 3-6 was comparable to the other two acids (53-65%). Treatments with HCl had better overall color retention in high acidity solutions when compared with both citric and formic acids.

The abundance of H^+ in pH 1 solutions stabilizes the flavylium cation, and contributes to color stability. However the stability of pigments in formic acid solutions

was lower than the other two acids at pH 1 and pH 2. This may be because of the reducing properties of formic acid (*194*). As a reducing agent, at high concentrations, formic acid may get involved with redox reactions of the pigments, hence negatively affecting the thermal stability of 3-deoxyanthocyanins.

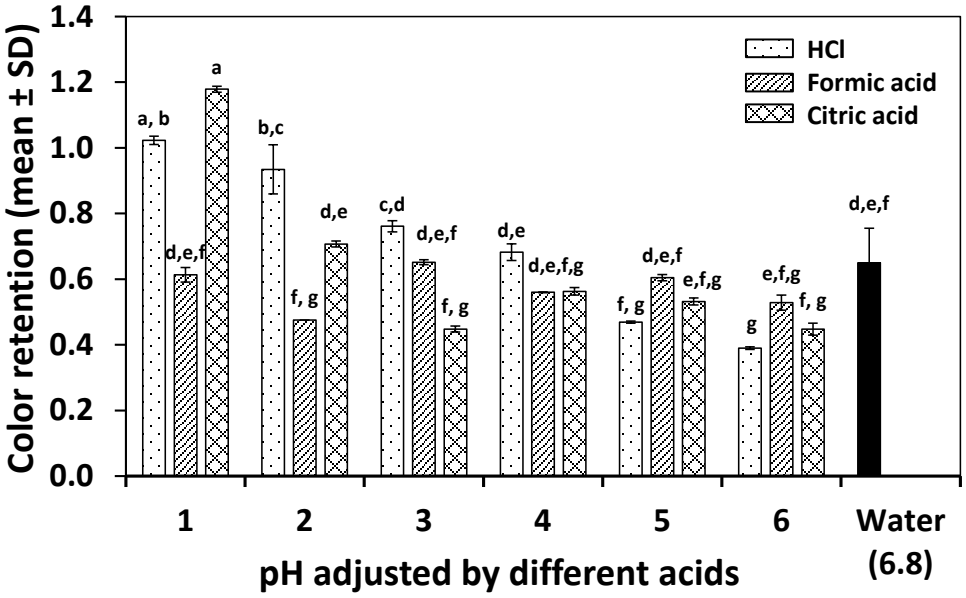


Figure 30. Color retention measured at λ_{max} of crude 3-deoxyanthocyanin extract after heat treatment, at pHs 1-6 and neutral aqueous solutions. Data are expressed as mean ± SD of duplicate runs. Treatments with the same letter do not significantly differ (Tukey's HSD, $p < 0.05$). Color retention was defined as absorbance of heat treated sample compared with corresponding non-heated control.

Many studies have explored the stability of anthocyanins from different sources, but mostly at temperatures below 100 °C. Mishra et al. (*195*) reported that grape pomace retained 15-17% anthocyanins after heating in steel cans in a steam retort at 126.7 °C (steam temperature) for 25 min. Jimenez et al. (*196*) found that the color of reconstituted blackberry juice (pH 2.8) retained about 25% after heating at 120 °C for 33

min. The crude 3-deoxyanthocyanin extract showed higher overall color retention after similar temperature-time treatment, which suggests better thermal stability. Thus the sorghum pigments may serve as functional food colorants.

Thermal Stability of 3-Deoxyanthocyanidins: Degradation Mechanism and Structure-Stability Relationship

The color retention of apigeninidin, luteolinidin and their methoxylated derivatives after heat treatment was determined in pHs 1-6 and neutral aqueous solutions to evaluate potential impact of 3-deoxyanthocyanin composition on sorghum pigment stability. Similar to the crude 3-deoxyanthocyanin extract, pH and acidulants affected the color retention of the pure compounds after heat treatment. HCl and citric acid treatments showed a similar trend: Highest stability at pH 1 and pH 2, with > 80% color retention (Figure 31). At pHs 3-6, the color retention was relatively similar among treatments with values of 68-78%. As observed for the crude 3-deoxyanthocyanin extract, formic acid treatments showed a completely different trend: Color was least retained at pH 1 (7.8-46%), followed by pH 2 (26-60%). Treatments at the other pH values had similar retention as HCl and citric acids (64-79%). The reducing properties of formic acid may have negatively affected color retention in pH 1 and 2 formic acid solutions (*194*). Samples treated in neutral aqueous solutions showed similar stability as the pHs 3-6 treatments, with 68-84% color retained.

The 3-deoxyanthocyanidins showed greater color retention after heat treatment compared with the crude 3-deoxyanthocyanin extract in general (Figure 30 and Figure

31). This was somewhat unexpected. The changes to other polyphenolic compounds in the crude 3-deoxyanthocyanin extract during heat treatment, such as oxidation, may have triggered degradation reactions of the pigments, hence reduced the relative color stability of the crude 3-deoxyanthocyanin extract. It will be important to study the thermal stability of additional natural 3-deoxyanthocyanin extracts with different pigment and copigment composition, especially those ones from various non-grain plant tissues, such as glumes, leaves, and leaf sheaths. This will be helpful to select suitable pigments and obtain them from most economical sources.

The color retention of 3-deoxyanthocyanidins was also significantly affected by molecular structure. This effect was most apparent in formic acid treatments at low pH values (1 and 2). The methoxylated 3-deoxyanthocyanidins were less stable compared with non-methoxylated ones, with color retention of 7.8-39% versus 45-60%, respectively. The degree of methoxylation did not affect color retention: mono-methoxylated apigeninidin (5- and 7-OMe-APG) had 7.8-39% color retained while di-methoxylated ones (5,7-OMe-APG and 5,7-OMe-LUT) had 19-34%. However, the position of *O*-methyl substitution seems to affect stability: 5-OMe-APG was the only sample which has a single C-5 *O*-methyl substitution and yet had the lowest stability among all 3-deoxyanthocyanidin compounds (7.8% and 26% in pH 1 and pH 2, respectively). Since C-5 position is the most reactive one in anthocyanins and 3-deoxyanthocyanins (*197*), the lower stability of C-5 *O*-methyl substituted compounds may be due to enhanced degradation reactions at this position because of the $–OCH_3$ group.

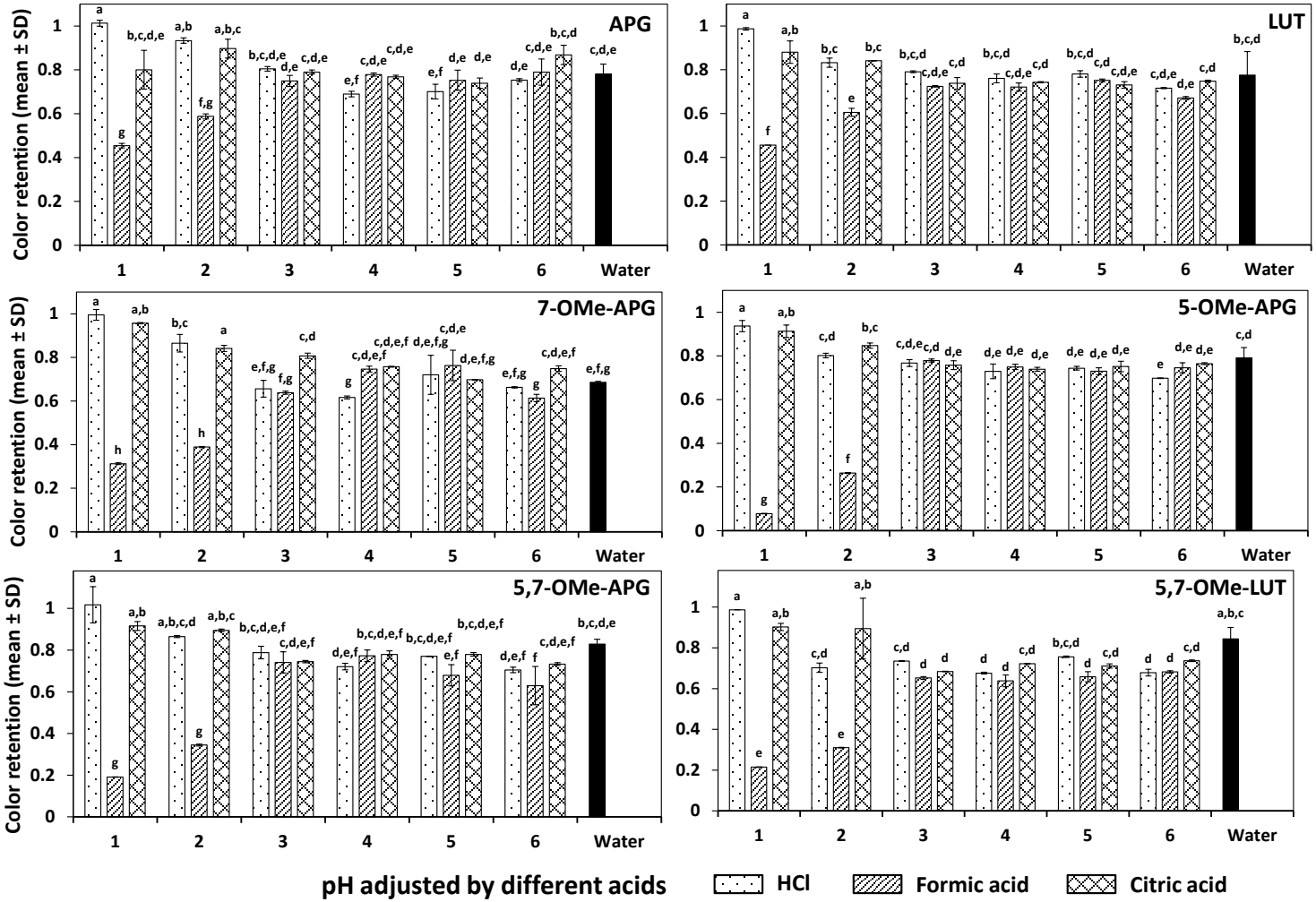


Figure 31. Color retention measured at λ_{max} of 3-deoxyanthocyanidins after heat treatment, in pHs 1-6 and neutral aqueous solutions. Treatments within the same compound with the same letter do not significantly differ (Tukey's HSD, $p < 0.05$). Color retention was defined as absorbance of heat treated sample compared with corresponding non-heated control.

Heat Induced Structural Changes to 3-Deoxyanthocyanidins

In order to elucidate the structural changes contributing to loss of 3-deoxyanthocyanidin color intensity, HPLC profiles were obtained on all treatments. For all three acids, structural changes at pHs 3-6, as well as that in neutral conditions, were very similar. Thus chromatograms from a subset of representative samples (APG, 5-OMe-APG, and 5,7-OMe-APG) treated with all three acids are shown as examples of degradation pattern (Figures 32-34). Structures of degradation products in all these treatments were determined by UPLC-MS using neutral solutions as the representative sample. For treatments at low pH conditions (pH 1 and 2), structure changes were minimal except for formic acid treatments. Figures 35-37 show chromatograms of the same subset (APG, 5-OMe-APG, and 5,7-OMe-APG) of samples treated with all three acids at pH 1. Structures of degradation products due to formic acid treatment (pH 2 as an example) were determined by UPLC-MS. Table 19 summarizes the products generated after heat treatment detected by UPLC-MS in neutral (water) and formic acid pH 2 solutions. Table 20 summarizes the MS/MS fragmentation patterns of key degradation products.

In general, demethylation of 5-OCH_3 substituted and condensation of 5-OH substituted 3-deoxyanthocyanidins were observed. Both reactions generated 3-deoxyanthocyanidin derivatives which still had absorbance between 470-490 nm. Formation of chalcones was identified as a common and major degradation pattern in all samples, moreover, formation of reduced chalcones was identified as a minor degradation product in neutral conditions and the most significant product in formic acid

low pH treatments (1 and 2). Minor peaks of C-ring fragmentation products and chalcone adducts were also detected.

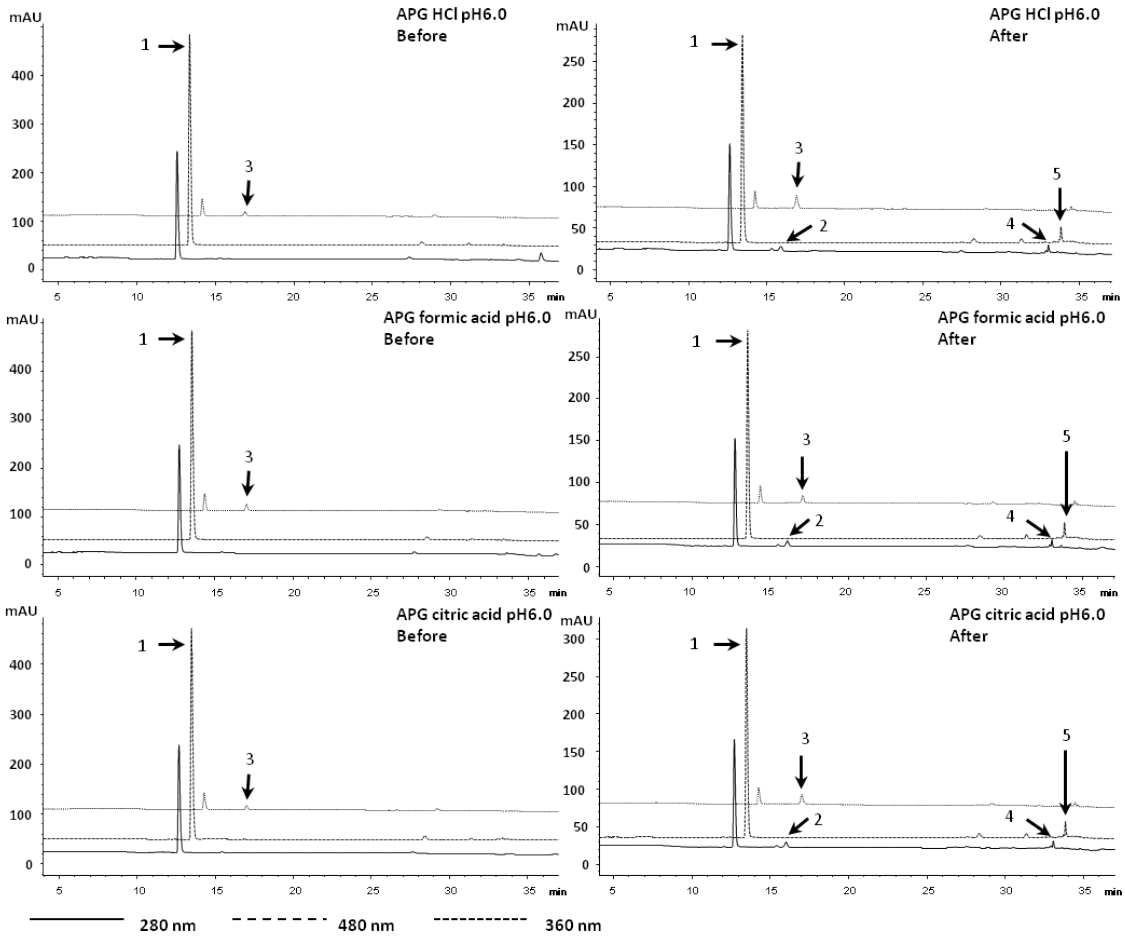


Figure 32. HPLC chromatograms of apigeninidin (APG) in pH 6 aqueous solutions before and after heat treatment. Chromatograms at 280 nm, 480 nm, and 360 nm are sequentially stacked in one figure with 2% offset in retention time and 10% offset in absorbance. Peak numbers are referenced to Table 19.

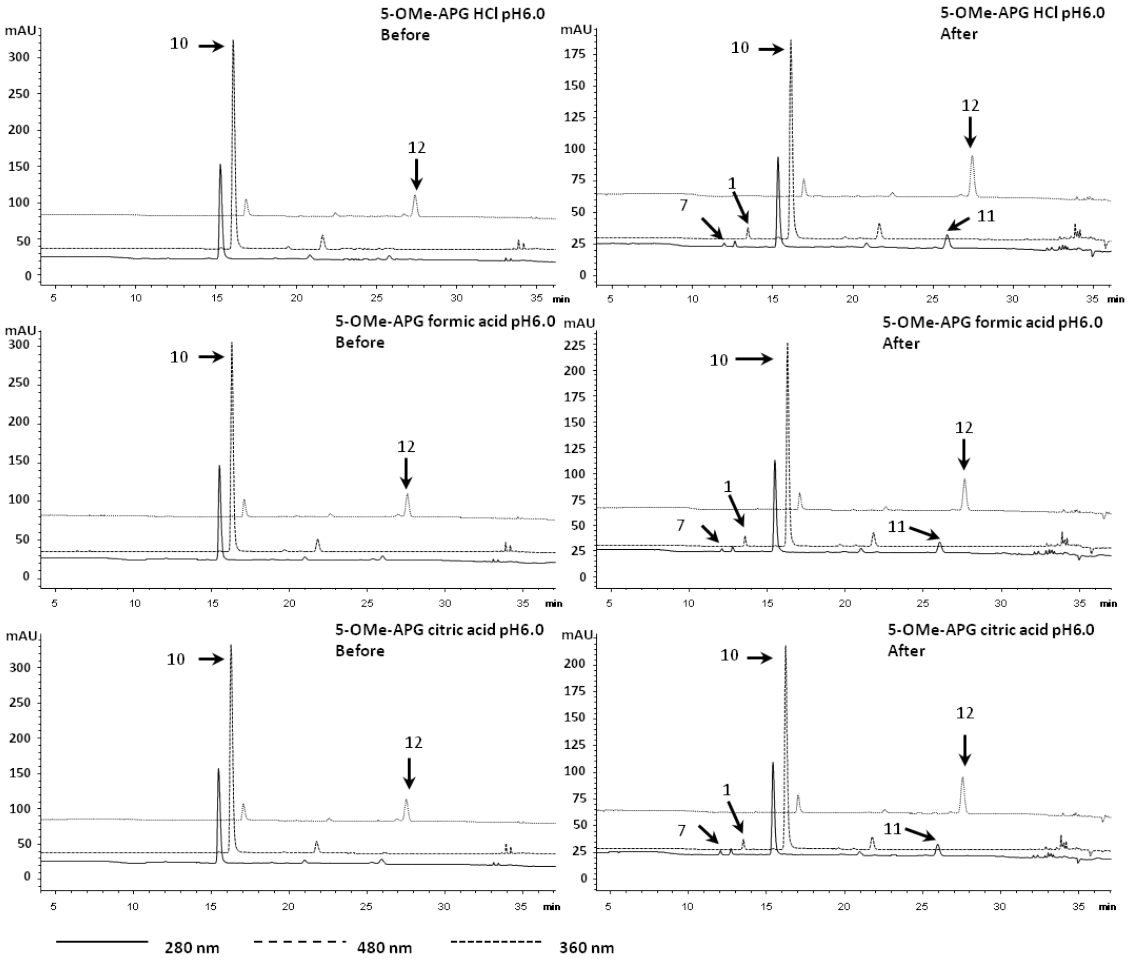


Figure 33. HPLC chromatograms of 5-*O*-methyl-apigeninidin (5-OMe-APG) in pH 6 aqueous solutions before and after heat treatment. Chromatograms at 280 nm, 480 nm, and 360 nm are sequentially stacked in one figure with 2% offset in retention time and 10% offset in absorbance. Peak numbers are referenced to Table 19.

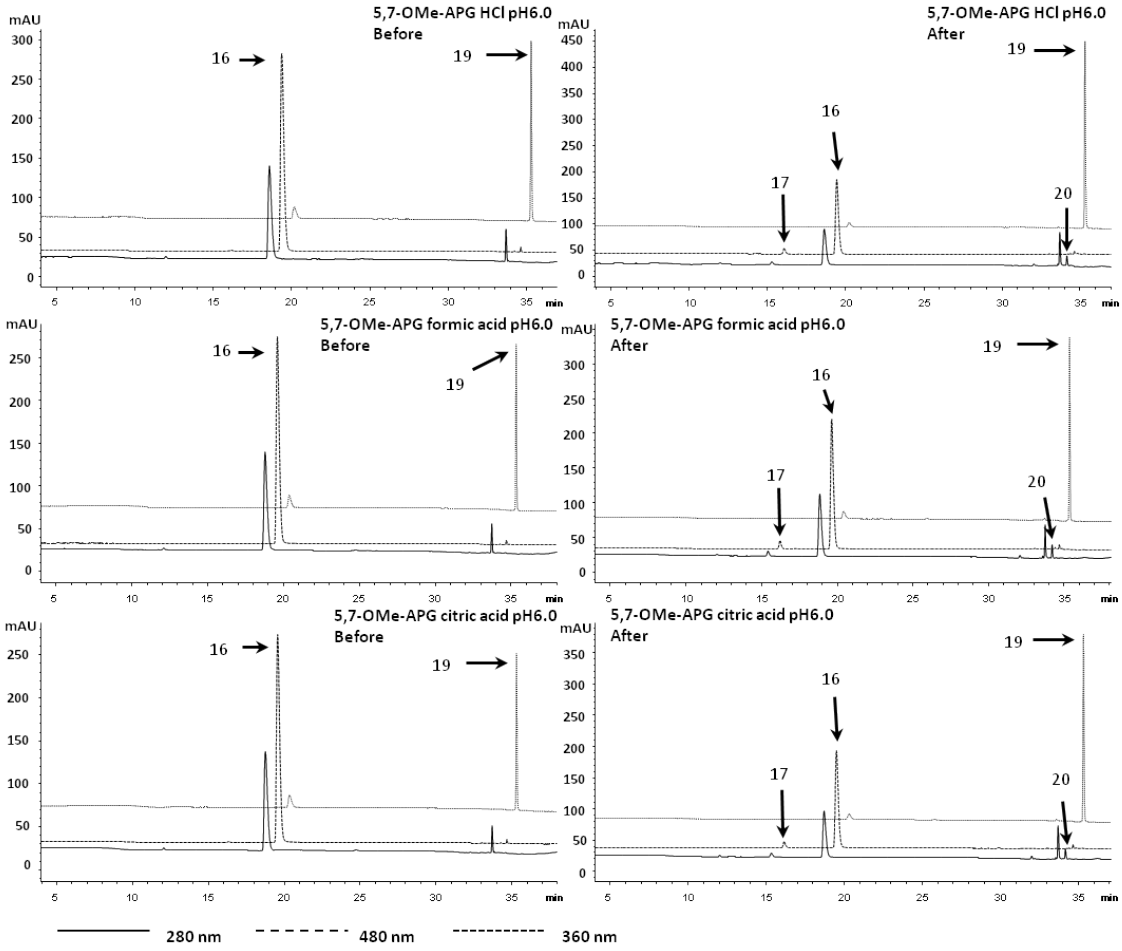


Figure 34. HPLC chromatograms of 5,7-*O*-methyl-apigeninidin (5,7-OMe-APG) in pH 6 aqueous solutions before and after heat treatment. Chromatograms at 280 nm, 480 nm, and 360 nm are sequentially stacked in one figure with 2% offset in retention time and 10% offset in absorbance. Peak numbers are referenced to Table 19.

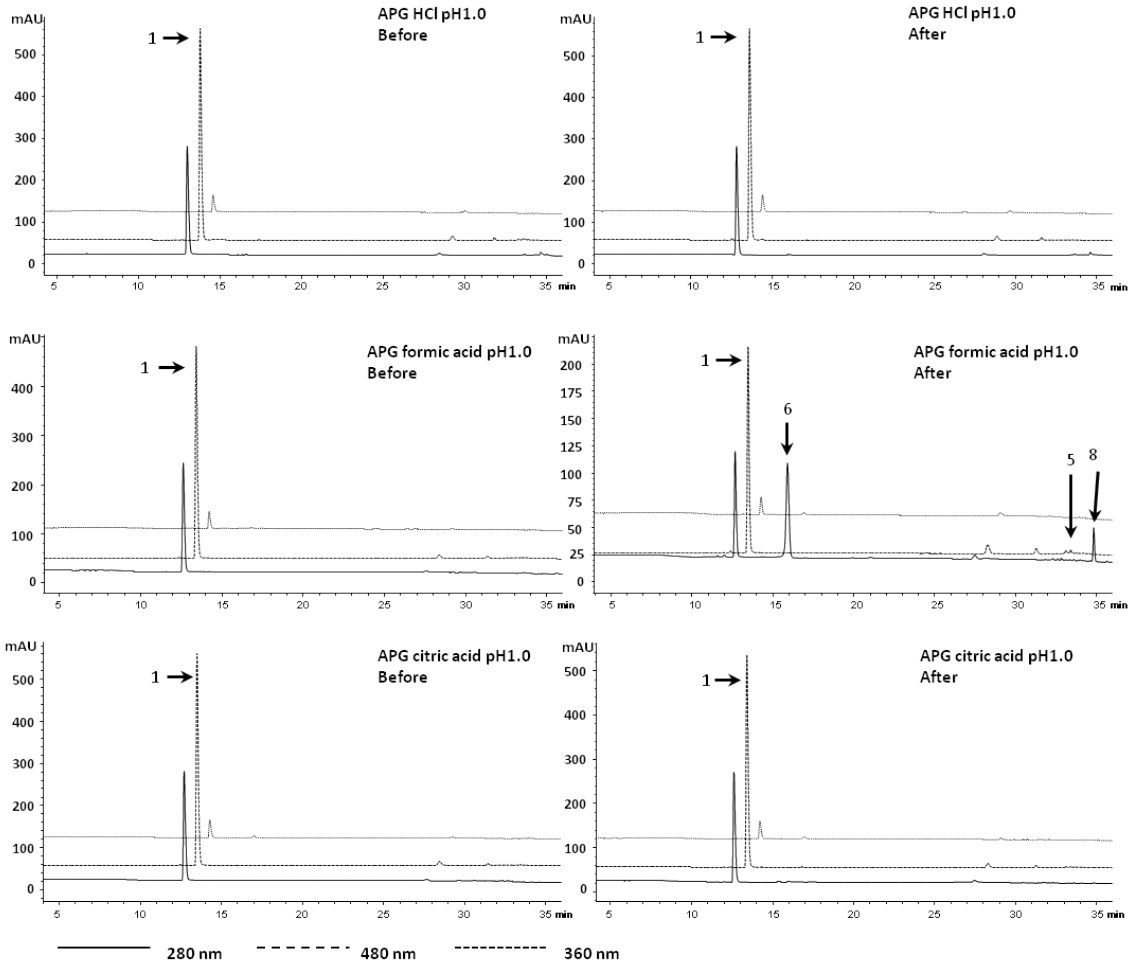


Figure 35. HPLC chromatograms of apigeninidin (APG) in pH 1 aqueous solutions before and after heat treatment. Chromatograms at 280 nm, 480 nm, and 360 nm are sequentially stacked in one figure with 2% offset in retention time and 10% offset in absorbance. Peak numbers are referenced to Table 19.

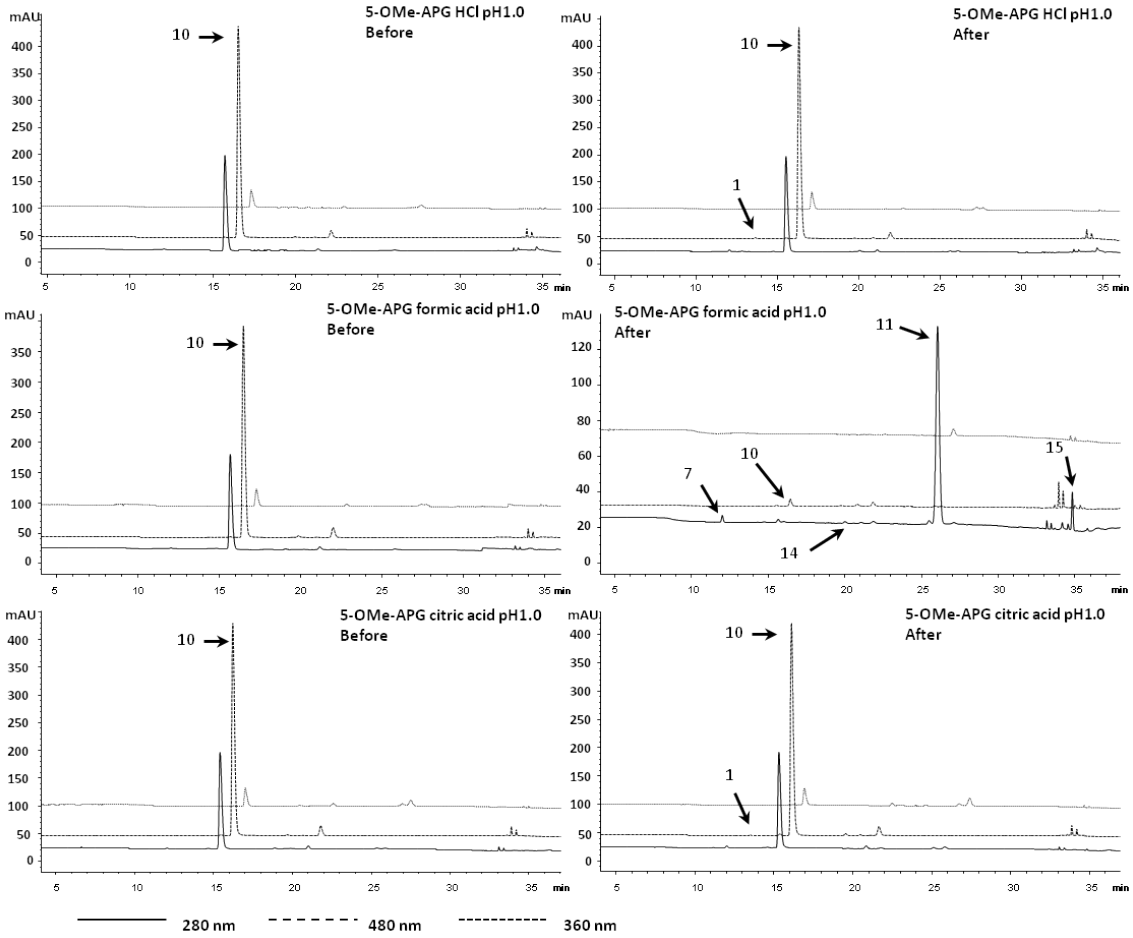


Figure 36. HPLC chromatograms of 5-*O*-methyl-apigeninidin (5-OMe-APG) in pH 1 aqueous solutions before and after heat treatment. Chromatograms at 280 nm, 480 nm, and 360 nm are sequentially stacked in one figure with 2% offset in retention time and 10% offset in absorbance. Peak numbers are referenced to Table 19.

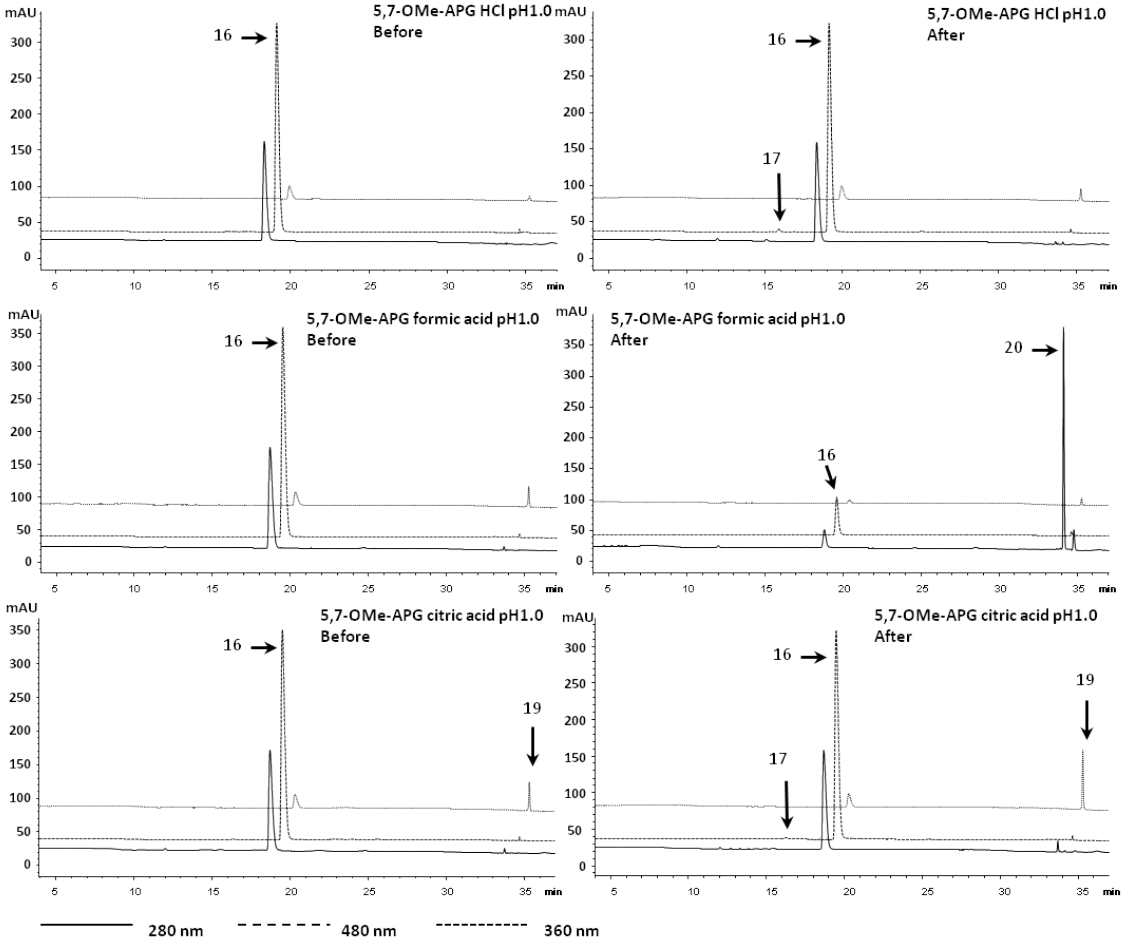


Figure 37. HPLC chromatograms of 5,7-*O*-methyl-apigeninidin (5,7-OMe-APG) in pH 1 aqueous solutions before and after heat treatment. Chromatograms at 280 nm, 480 nm, and 360 nm are sequentially stacked in one figure with 2% offset in retention time and 10% offset in absorbance. Peak numbers are referenced to Table 19.

Table 19. Identification of compounds detected after heat treatment of 3-deoxyanthocyanidins in water (neutral) and formic acid pH 2 (acidic) aqueous solutions (monitored at 280 nm, 340 nm, and 485 nm) based on UPLC retention time (t_R), UV-*vis* spectroscopic characteristics (λ_{max}), and MS spectroscopic pattern. Ionization was performed in the positive mode for 3-deoxyanthocyanins and in the negative mode for the other compounds.

Compound and conditions	**Peak No.**	**t_R (min)**	**λ_{max} (nm)**	**$[M+H]^+$ (*m/z*)**	**$[M-H]^-$ (*m/z*)**	**Proposed Identification[a]**
Apigeninidin (APG)	1	4.41	471	255	253	Standard
neutral	2	5.589	277		273	Reduced APG chalcone
	3	5.666	375		271	APG chalcone
	4	15.467	224/372		525	Adduct 1
	5	17.32	471	509		APG dimer
	6	17.613	365		525	Adduct 2
acidic	7	3.371	282		135[b]	1-(4-hydroxyphenyl)ethanone
	2	5.596	277		273	Reduced APG chalcone
	8	15.181	264		527	Adduct 3
	9	15.482	224/372		525	Adduct 4
	5	17.32	471	509		APG dimer
5-OMe-APG	10	6.31	474	269	267	Standard
neutral	1	4.41	471	255		APG
	11	9.932	266/451		287	Reduced 5-OMe-APG chalcone
	12	10.298	375		285	5-OMe-APG chalcone
	13	15.252	269/477		535	Adduct 5
acidic	7	3.374	276		135	1-(4-hydroxyphenyl)ethanone
	1	4.41	471	255		APG
	14	7.354	294		167	Methoxylated phloroglucinaldehyde
	11	9.932	277		287	Reduced 5-OMe-APG chalcone
	15	15.17	263	523		Adduct 6
5,7-OMe-APG	16	7.25	472	283	281	Standard
neutral	17	5.83	469	269		7-OMe-APG
	19	15.858	372		299	5,7-OMe-APG chalcone
	20	16.345	276		301	Reduced 5,7-OMe-APG chalcone
acidic	21	1.996	255		137	*p*-Hydroxybenozic acid
	17	5.83	469	269		7-OMe-APG
	20	16.337	276		301	Reduced 5,7-OMe-APG chalcone

Table 19-Continued

Compound and conditions	Peak No.	t_R (min)	λ_{max} (nm)	$[M+H]^+$ (*m/z*)	$[M-H]^-$ (*m/z*)	Proposed Identification
Luteolinidin (LUT)		3.363	487	271	269	Standard
neutral		3.704	278/306		289	Reduced LUT chalcone
		3.953	379		287	LUT chalcone
acidic		3.704	278/306		289	Reduced LUT chalcone
		3.953	379		287	LUT chalcone
		10.976	277/308		559	Adduct 7
7-OMe-APG		5.83	469	269	267	Standard
neutral		3.363	278		135	1-(4-hydroxyphenyl)ethanone
		10.196	275		287	Reduced 7-OMe-APG chalcone
		17.81	486	537		7-OMe-APG dimer
		22.06	486	805		7-OMe-APG trimer
acidic		3.374	276		135	1-(4-hydroxyphenyl)ethanone
		9.514	373		285	7-OMe-APG chalcone
		10.204	277		287	Reduced 7-OMe-APG chalcone
		17.81	486	537		7-OMe-APG dimer
		22.06	486	805		7-OMe-APG trimer
		22.803	273		555	Adduct 8
5,7-OMe-LUT		6.26	487	299	297	Standard
neutral		1.333	218/258/ 293		153	Phloroglucinaldehyde
		4.66	487	285		7-OMe-LUT[c]
		13.28	377		315	5,7-OMe-LUT chalcone
acidic		4.66	487	285		7-OMe-LUT[c]
		13.173	276/309/ 377		317	Reduced 5,7-OMe-LUT chalcone
		13.281	377		315[d]	5,7-OMe-LUT chalcone

[a]Refers to UPLC-MS-MS/MS spectroscopic patterns summarized in Table 20. [b]Dominant MS fragment. [c]Assignment of *O*-methyl substitution pattern was based on degradation pattern of 5,7-OMe-APG. [d]Co-eluting, very small peak.

Table 20. Identification of compounds detected after heat treatment of 3-deoxyanthocyanidins (monitored at 280 nm, 340 nm, and 485 nm) based on UPLC retention time (t_R), UV-*vis* spectroscopic characteristics (λ_{max}), and MS-MS/MS spectroscopic pattern. Ionization was performed in the negative or positive mode.

Peak No.	**t_R (min)**	**λ_{max} (nm)**	**$[M+H]^+$ (*m/z*)**	**$[M-H]^-$ (*m/z*)**	**MS/MS fragments (*m/z*)**	**Proposed Identification**
7	3.37	276		135	120, 93, 92	1-(4-hydroxyphenyl)ethanone
14	7.35	294		167	152 , 124, 96	Methoxylated phloroglucinaldehyde
	9.51	373		285	191, 165, 93	7-OMe-APG chalcone
11	9.93	277		287	193, 151, 147, 139, 93	Reduced 5-OMe-APG chalcone
	10.20	277		287	193, 191, 151, 147, 139	Reduced 7-OMe-APG chalcone
15	15.17	263	523		507, 491, 415, 370, 255	Adduct 6
	22.06	486	805		537, 385, 269	7-OMe-APG Trimer
	22.80	273		555	267	Adduct 8

Compounds generated by demethylation. All the 5-*O*-methyl substituted 3-deoxyanthocyanidins (5-OMe-APG, 5,7-OMe-APG, and 5,7-OMe-LUT) generated new pigment peaks with λ_{max} between 470-490 nm that eluted earlier than the parent compound (Figures 33 and 34). By comparing the masses and retention times with those of the standards, it was confirmed that all the 5-*O*-methyl substituted compounds were partially demethylated at position C-5 into their corresponding 5-OH substituted compounds (Table 19, Figure 38). The fact that C-7 *O*-methyl substituted compound (7-OMe-APG) was not demethylated, may be due to the higher reactivity of the C-5 than C-7 as reported for anthocyanins (*197*). This indicates 3-deoxyanthocyanin structure may affect thermal stability.

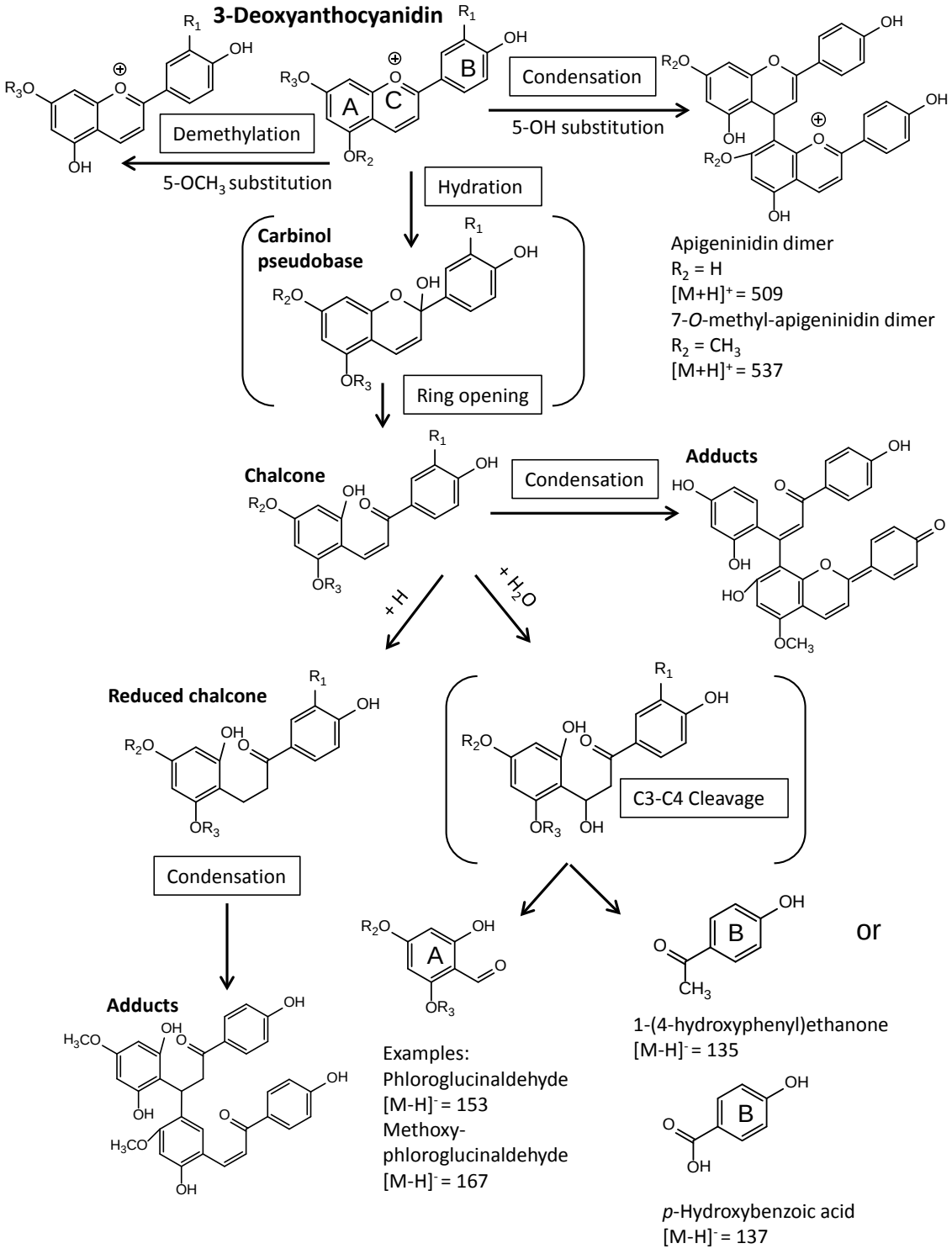


Figure 38. A schematic illustration of thermal degradation mechanism of 3-deoxyanthocyanidins. Intermediates [in brackets] were not detected.

Compounds generated by condensation reaction. Two C-5 –OH substituted 3-deoxyanthocyanidins, APG and 7-OMe-APG formed condensed pigments which had similar λ_{max} as 3-deoxyanthocyanins and eluted much later than the parent compounds (Figure 32). The product generated from APG (t_R = 17.322 min, λ_{max} = 471 nm) had $[M+H]^+$ of *m/z* 509, and the one from 7-OMe-APG (t_R = 17.806 min, λ_{max} = 486 nm) had $[M+H]^+$ of *m/z* 537. These two peaks had elution profiles and masses that matched dimers of APG and 7-OMe-APG, respectively (*193*) (Figure 38). 7-OMe-APG also generated a pigment product (t_R = 22.064 min, λ_{max} = 486 nm) which had $[M+H]^+$ of *m/z* 805 and had major MS/MS fragments at *m/z* 537 (49% intensity, M-268 amu = loss of a 7-OMe-APG group), *m/z* 269 (13% intensity, M-536 amu = loss of a 7-OMe-APG dimer group), and *m/z* 385 (45% intensity) (Table 20), which matched the fragmentation pattern for 7-OMe-APG trimer. Interestingly, the condensed dimers and trimers were only detected in APG and 7-OMe-APG samples after heat treatment, but not in the 5-*O*-methyl substituted samples. This indicates that the 5-OH structure allows easier formation of the C4-C8 linkage of the dimers and trimers. This 5-OH reaction pattern was also observed in cyclic condensation reactions between 3-deoxyanthocyanidins and pyruvic acid (*198*).

Anthocyanins from various sources, such as grape pomace (*164*), blackberry juice (*199*), wine (*200*), black olives (*201*), condense during processing and storage. Due to the presence of other phenolic compounds, flavanol-anthocyanin and pyrano-anthocyanin type dimeric or oligomeric products are commonly detected. These condensed products are considered to contribute to stability of color during long term

storage. The formation of condensed dimers and trimers from 5-OH substituted 3-deoxyanthocyanins would possibly contribute to color retention and stability after heat treatment.

Chalcone formation after heat treatment. Products with λ_{max} at 370-379 nm and a pattern of $[M-H]^-$ of *m/z* [M+18 amu] were detected in all samples after heat treatment at neutral and pHs 3-6 (Figures 32-34, Table 19). For example, the [M+18 amu] product from 7-OMe-APG (t_R = 9.51 min, λ_{max} = 373 nm, $[M-H]^-$ = 285) had MS/MS fragments of *m/z* 165 (dominant ion), *m/z* 191 (37% intensity), and *m/z* 93 (43% intensity) (Table 20). The fragmentation pattern matched that of 7-OMe-APG chalcone (Figure 39). Chalcone is an opened C-ring product of 3-deoxyanthocyanidin as a result of hydroxyl addition at C-2 and corresponding formation of carbinol pseudobase in mildly acidic aqueous solutions (*202*). Based on the spectrophotometric characteristics and MS pattern, chalcones of LUT, APG, 7-OMe-APG, 5-OMe-APG, 5,7-OMe-APG, and 5,7-OMe-LUT were all identified (Table 19).

Chalcones were detected as the major degradation product in samples heated in neutral and pHs 3-6 solutions. The type of acidulant did not have a significant effect on the extent of chalcone formation (Figures 32-34).

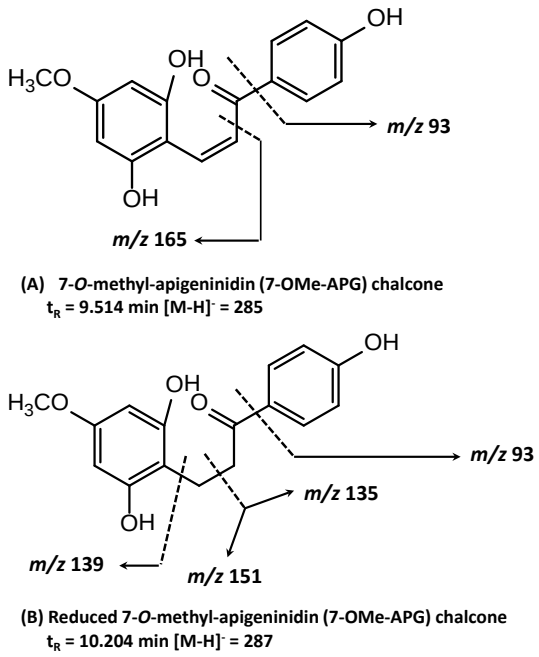


Figure 39. Chemical structures and schematic fragmentation pattern (referenced to Table 20) of 7-*O*-methyl-apigeninidin chalcone (A) and reduced chalcone (B).

Structure of 3-deoxyanthocyanidins had an effect on the formation of chalcone. Non-methoxylated 3-deoxyanthocyanidins, APG and LUT, formed very small quantities of chalcones, while degree of methoxylation increased chalcone formation due to heat treatment (Figures 31-33). At pHs 4-6 and neutral conditions, chalcones were detected before heat treatment as a result of spontaneous hydration reaction at C-2 position.

3-Deoxyanthocyanidins have greater deprotonation rate constant (k_a) than hydration rate constant (k_h) (*202*), hence convert less to the colorless carbinol pseudobases in aqueous solutions, which would lead to less formation of chalcones at low acidity and after heat treatment. Formation of carbinol pseudobase due to hydrophilic attack at the C-2 position, and corresponding formation of chalcones is the

first step of anthocyanins degradation due to thermal treatment (*203-205*). In this regard, less formation of chalcones of 3-deoxyanthocyanins may have contributed to better stability after heat treatment compared to anthocyanins.

The methoxylated 3-deoxyanthocyanidins showed less color retention after heat treatment (Figures 31, 33, 34), which may be because of their higher tendency to form chalcones and hence are more prone to thermal degradation. Moreover, degree of methoxylation seemed to further increase the level of chalcone formation (Figures 32-34), which was probably the reason for lower color retention of 5,7-OMe-APG and 5,7-OMe-LUT in pHs 4-7. C-5 and C-7 *O*-methyl substitution eliminates the electron-donating capacity of –OH at these positions compared to APG and LUT, thus strongly favors the cation hydration reaction towards more carbinol pseudobase and chalcone formation (*206*). Methoxylated anthocyanidins (such as malvidin, peonidin, petunidin) have been reported to form more chalcones than non-methoxylated ones (such as delphinidin and cyanidin) in pHs of 4-7 aqueous solutions (*207*). Furtado et al. (*203*) also found that malvidin had a slightly greater rate constant for chalcone formation and fission compared with cyanidin during thermal degradation. Thus, it is reasonable to believe that methoxylation of 3-deoxyanthocyanidins negatively affected their thermal stability.

Reduced chalcone formation after heat treatment. Products with absorbance (λ_{max}) at 265-276 nm and a pattern of $[M-H]^-$ of *m/z* [M+20 amu] were detected in all samples as major products after heat treatment in low pH formic acid solutions (pH 1 and 2), as well

as a minor product after heat treatment in higher pH solutions (Table 19, Figures 32-37).

Peak 11 (t_R = 9.932 min, λ_{max} = 277 nm, $[M-H]^-$ = 287) was found as a product of 5-OMe-APG after heat treatment, which had MS/MS fragments of *m/z* 139 (dominant ion), *m/z* 135, *m/z* 151, and *m/z* 93 (Table 3); a similar compound (t_R = 10.196 min, λ_{max} = 275 nm, $[M-H]^-$ = 287) with almost identical fragmentation pattern was found as a product of 7-OMe-APG. The fragmentation pattern (Figure 39) of these two compounds was similar as that of 3-deoxyanthocyanin chalcones, but according to mass and UV-*vis* spectroscopic characteristics, the structures must have a saturated C3-C4 bond; thus were denoted as reduced chalcones. Based on the spectrophotometric characteristics and MS pattern, reduced chalcones from LUT, APG, 7-OMe-APG, 5-OMe-APG, 5,7-OMe-APG, and 5,7-OMe-LUT were identified (Table 19). Adams (*208*) first identified α-diketone as a thermal degradation product of cyanidin, which was formed as a result of formation of chalcones. The detection of reduced chalcones in 3-deoxyanthocyanidins after heat treatment in pHs 4-6 solutions followed a similar chalcone → ketone reaction pattern.

In samples treated in low pH formic acid solutions (pH 1 and 2), reduced chalcones were the major product formed after heat treatment. This effect was unique to formic acid, and only at pH 1 and 2. Formic acid has been used as a reducing agent for its capacity to give out both hydrogens and decompose into carbon dioxide (*194*). Hence the favored formation of reduced chalcones in pH 1 and 2 formic acid solutions should be a result of the reducing capacity of formic acid. The relative quantities of reduced chalcones followed the same trend as for formation of chalcones: Methoxylation

increased the level of reduced chalcones formed. This was probably due to the higher tendency of methoxylated 3-deoxyanthocyanidins to form chalcones (*203, 207*). Based on this reaction pattern, we thus conclude that the reduced color intensity of 3-deoxyanthocyanidins in pH 1 and 2 formic acid solutions is probably due to the shift of equilibration to formation of reduced chalcones, which would favor higher chalcone formation from flavylium cation.

C-Ring fragments and adducts formation after heat treatment. Several peaks with various masses were detected in samples after heat treatment. MS pattern, elution pattern, and the spectrophotometric characteristics indicated some were fragments from C-ring fission due to thermal degradation.

Peak 7 (t_R = 3.374 min, λ_{max} = 276 nm, $[M-H]^-$ = 135) was identified as 1-(4-hydroxyphenyl)ethanone (Table 19 and Table 20, Figure 38), a B-ring fragment, based on degradation pattern of anthocyanidins (*158, 159*). **Peak 7** was detected in 7-OMe-APG neutral and formic acid pH 2 solutions, as well as 5-OMe-APG formic acid pH 2 solution after heat treatment (Table 19). Similarly, *p*-hydroxybenzoic acid (t_R = 1.996 min, λ_{max} = 255 nm, $[M-H]^-$ = *m/z* 137) was identified as another B-ring fragment (Figure 38), in 5,7-OMe-APG formic acid pH 2 solution after heat treatment.

Peak 14 (t_R = 7.354 min, λ_{max} = 294 nm, $[M-H]^-$ = 167) was identified as 2-methoxy-phloroglucinaldehyde (Table 20, Figure 38), an A-ring fragment, according to patterns of anthocyanidins (*158, 159*). **Peak 14** was only detected in 5-OMe-APG formic acid pH 2 solution after heat treatment. Similarly, phloroglucinaldehyde (t_R = 1.333 min,

λ_{max} = 218/258/293 nm, $[M-H]^-$ = *m/z* 153) was also detected in 5,7-OMe-LUT formic acid pH 2 solution after heat treatment (Table 19, Figure 38).

Formation of C-ring cleavage products is a common degradation scheme of anthocyanidins. Products such as phloroglucinaldehyde, *p*-hydroxybenzoic acid and its hydroxylated or methoxylated derivatives have been reported in different studies (*158, 159, 203*). Formation of chalcone is considered as the initial step for anthocyanidins to yield C-ring cleavage products. All C-ring cleavage products were detected as minor peaks in pH 1 and 2 formic acid solutions, especially in the methoxylated 3-deoxyanthocyanidins. Thus without the reducing effect of formic acid in these reactions, the evidence actually suggests that 3-deoxyanthocyanidins resisted fragmentation during thermal treatment.

Minor dimeric adducts were detected in most samples after heat treatment except the two dimethoxylated 3-deoxyanthocyanidins (adduct 1-adduct 8, Table 19). These were likely chalcone adducts based on UV-*vis* characteristics, MS pattern and MS/MS fragmentation pattern. Examples of fragmentation pattern and proposed structures are shown in Figures 40 and 41 for adduct 6 (**peak 15**) and adduct 8 (chromatograms not shown), respectively.

Sadinova et al. (*158, 159*) observed several adducts after heat treatment of purified anthocyanin fractions from different fruits/vegetables. They deduced the adducts were from two fragments from either the A-ring or the B-ring. Interestingly, the adducts detected in our study were mostly condensed with intact C-rings. This may indicate higher resistance to C-ring cleavage of 3-deoxyanthocyanidins than

anthocyanidins during heat treatment, which may additionally contribute to their thermal stability.

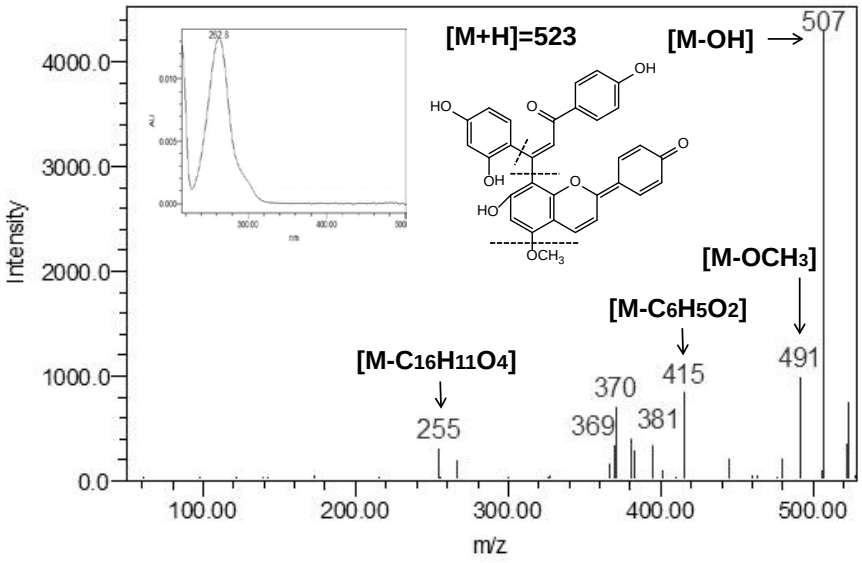


Figure 40. Proposed structure of adduct 6 (**peak 15**), as well as UV-*vis* characteristics and MS/MS fragmentation pattern.

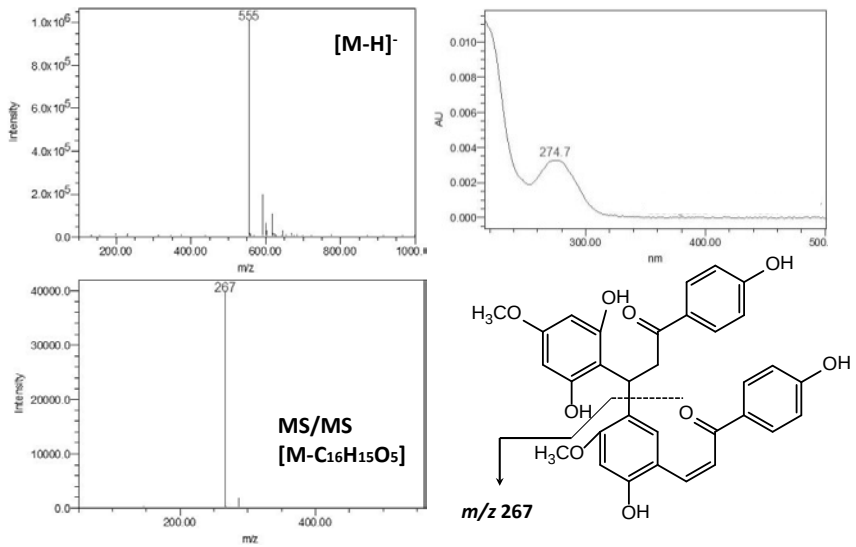


Figure 41. Proposed structure of adduct 8 (peak not shown), as well as UV-*vis* characteristics and MS/MS fragmentation pattern.

Overview of Thermal Degradation Mechanisms of 3-Deoxyanthocyanidins

In summary, the degradation mechanisms of 3-deoxyanthocyanidins were depended on pH and acidulants, as well as the structure. Only the C-5 -OH substituted 3-deoxyanthocyanidins (APG and 7-OMe-APG) condensed into dimers and trimer. The C-5 *O*-methyl substituted 3-deoxyanthocyanidins (5-OMe-APG, 5,7-OMe-APG, and 5,7-OMe-LUT) demethylated into their corresponding 5-OH substituted 3-deoxyanthocyanidins. This was due to the more reactive nature of C-5 relative to C-7 (*197*). Chalcone formation was the major color degradation product after heat treatment in pHs 3-6 and neutral aqueous solutions. High formic acid environment (pH 1 and 2) favored the formation of reduced chalcones, which shifted equilibration towards 3-deoxyanthocyanidin hydration and chalcone formation thus resulted in greater color loss, due to the reducing capacity of formic acid (*194*). The C-ring cleavage products following chalcones or reduced chalcones formation were detected as minor peaks mostly in the methoxylated 3-deoxyanthocyanidins in the low pH formic acid solutions. Minor adducts of chalcones were also detected, especially in the low pH formic acid solutions. The thermal degradation pattern of 3-deoxyanthocyanidins followed the same reaction identified for the anthocyanidins. Compared with anthocyanidins, 3-deoxyanthocyanidins were more resistant to C-ring fission, and chalcone formation was the major degradation product, which indicated relatively good thermal stability.

Conclusions

The fact that no meaningful changes in color retention of sorghum 3-deoxyanthocyanidins could be detected after heating in 95 °C water bath for up to 2 hr (data not shown) indicated good thermal stability of these pigments. In the subsequent treatments under severe heat (121 °C 30 min under pressure), it was clear that pH as well as acidulants affected thermal stability of 3-deoxyanthocyanidins. All 3-deoxyanthocyanidins retained good color stability (< 20% loss) at pH 1 and 2 in the presence of HCl and citric acid, and retained 64-84% color in pHs 3-6 for all acidulants, as well as water after heat treatment. The effect of pH on color stability was expected because low pH conditions favor stability of colored 3-deoxyanthocyanidin flavylium cations.

The type of acidulants did not affect color retention significantly at pHs 3-6, however, contrary to the effect of HCl and citric acid, formic acid treatments at pH 1 and pH 2 negatively affected the color retention of 3-deoxyanthocyanidins, due to its reducing capacity and generation of reduced 3-deoxyanthocyanidin chalcones. This suggests that the characteristics of acidulants should be considered during thermal processing of products containing natural anthocyanin pigments.

Structure of 3-deoxyanthocyanidins also had an impact on their thermal stability. Methoxylation of 3-deoxyanthocyanidins decreased the thermal stability, especially in pH 1 and 2 formic acid solutions. Hydroxylation on the B-ring did not seem to affect thermal stability of 3-deoxyanthocyanins. Chalcone formation was recognized as the

first step of thermal degradation. Methoxylation of 3-deoxyanthocyanidins increased the formation of chalcones, hence negatively affected thermal stability.

The presence of other phenolic compounds in the matrix of crude 3-deoxyanthocyanin extract may have affected the thermal stability of 3-deoxyanthocyanin pigments. More studies are needed to establish the effect of 3-deoxyanthocyanin and copigment composition on thermal stability of natural sorghum 3-deoxyanthocyanin pigment extracts.

Thermal stability of 3-deoxyanthocyanins is an important property when evaluating the potential of these pigments as bioactive food colorants. Results obtained from this study indicate good thermal stability of 3-deoxyanthocyanins, especially when tested as individual pure compounds. Strategies targeting less chalcone formations in low acid conditions, as well as the avoidance of reducing agents would help improve the stability of 3-deoxyanthocyanidins during thermal processing.

CHAPTER VI

SUMMARY

This study demonstrates that specific sorghum varieties possess estrogenic acitivity in cell models predominantly expressing ERα or ERβ. The estrogenic potential is related to phenolic compositions, especially the differences in flavonoid profiles among sorghum varieties. Flavones were found to be the most potent estrogenic compounds present in sorghum; while flavanones also suggested estrogenic activity at higher concentrations. On the other hand, studies with pure 3-deoxyanthocyanidins revealed that these pigments are probably not estrogenic. Experiments with pure apigenin (a flavone) and naringenin (a flavanone) supported the concept of higher estrogenic potency of flavones. In addition, we also found that naringenin enhanced the estrogenic potency of apigenin when the two compounds were combined at suboptimal levels. However, when combined at optimal levels, naringenin antagonized the estrogenic activity of apigenin. This evidence indicates that compounds in a crude mixture could synergistically exert bioactivity at lower concentrations than what is modeled with pure compounds. This study utilized crude sorghum phenolic extracts to determine the estrogenic activity, thus provided a more realistic evidence in the context of diet when compared to pure isolated compounds. Models using pure compounds may underestimate bioactivity by ignoring possible synergism among different polyphenols that occurs in a crude phenolic extract.

White and black sorghum extracts fed at 1% level in the diet inhibited the formation of premalignant lesions induced by carcinogen azoxymethane in the colons of ovariectomized mice. This suggested that estrogenic sorghum extracts may contribute to colon cancer prevention *in vivo* at concentrations achievable through diet. However, no changes were observed in the proportions of colonic apoptotic or proliferative cells of the mice fed with sorghum extracts compared with the control. This suggested that other mechanisms were likely responsible for the observed inhibition of premalignant lesion formation.

Thermal stability is an important property which could affect the chemopreventive capacity of sorghum phenolics. 3-Deoxyanthocyanins, as the most unique flavonoids in sorghum, have good potential to be used as functional food colorants. Sorghum 3-deoxyanthocyanins retained good color stability after heat treatment at 121 °C for 30 minutes (under pressure). The pH of media and type of acidulants affected color stability: More than 80% of color retained in pH 1 and 2 HCl and citric acid solutions, and 39-84% retained in solutions of pHs 3-7. Formic acid negatively affected the color stability at pH 1 and pH 2 due to its reducing capacity, while color retention in pHs 3-6 formic acid solutions was similar as HCl and citric acid. Methoxylation of 3-deoxyanthocyanidins decreased their color stability. Major degradation products of 3-deoxyanthocyanidins due to heat treatment were also identified, which would enable development of strategies to stabilize 3-deoxyanthocyanins during processing. This information would open up opportunities to

evaluate 3-deoxyanthocyanins as food colorants in the context of food products and to maximize their color stability during food processing.

Overall, the study showed that estrogenic activity of specific sorghum phenolic compounds is a likely mechanism for colon cancer prevention. This information is important in understanding the chemopreventive mechanisms of sorghum phenolic compounds, as well as identifying possible active components. The chemopreventive benefits of sorghum would promote the utilization of sorghum grains and brans as functional ingredients in food products. The 3-deoxyanthocyanins can be used as natural colorants in food products, especially low acid foods. The information on phenolic compositions of sorghum and estrogenic activity is also useful to selectively breed sorghum varieties accumulating compounds targeting prevention of specific diseases.

Further studies on estrogenic activity of sorghum phenolic compounds and colon cancer prevention could focus on:

1) Identifying molecular mechanisms for chemopreventive potential of sorghum polyphenols *in vivo*.

2) Understanding the interactions among different phenolic compounds in crude sorghum extracts on estrogenic activity, and the potential impact of these interactions on colon cancer prevention.

3) Determining active metabolites of sorghum phenolic compounds *in vivo* and their role on colon cancer chemoprevention.

4) Evaluating the effect of food processing on the bioactivity and bioavailability of sorghum phenolic compounds.

5) Assessing physiologically relevant dose of sorghum phenolic compounds for colon cancer prevention.

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